University of Alberta

GENETIC ANALYSIS OF THE *WEE1* KINASE OF *DROSOPHILA*

by

Donald Marshall Price ©

A thesis submitted to the faculty of Graduate studies and research in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

in

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ABSTRACT

The cell cycle is a descriptive term referring to the organization of events in the life of a (typically eukaryotic) cell. Securing the orderly succession of cell cycle events is crucial for maintaining genomic integrity. Failure to do so may result in cell death, impairment of the developmental program, and cellular transformation leading to cancer. In this thesis I describe genetic analyses of a cell cycle regulator, the *wee1* kinase of *Drosophila* (*Dwee1*). Wee1-like kinases are essential for checkpoint controls in the yeasts, and this thesis presents the first description of the role of this kinase in the development of a metazoan organism. Here I present a mutational study showing that the *Dwee1* gene has an essential maternal function controlling the timing of early embryonic nuclear divisions. In contrast, *Dwee1* function is dispensable for zygotic development of the animal, but is required for maintaining viability of larvae in the presence of hydroxyurea, an inhibitor of DNA replication. An eye-specific overexpression system for both Wee1-like kinases (*Dwee1* and *Dmyt1*) was developed, which produces a rough eye in adult flies. We observe that Glass Multimer Reporter (GMR)-driven *Dmyt1* interferes with the second mitotic wave cell division. This phenotype is subject to modification by altered levels of cell cycle regulators. We have used this overexpression system to explore known mutations, transgenes and deficiencies for genetic interactors (enhancers and suppressors) of the two Wee1-like kinases in *Drosophila*. Using this overexpression system, we observed genetic interactions consistent with a function for these genes in countering p53-

dependent cell death. Finally, we conducted an EMS mutagenesis screen for modifiers of these Wee1-like kinase-derived eye phenotypes. We observe that modulations of Notch signaling modify the *GMR-Dmyt1* phenotype, thus raising the possibility that *Dmyt1* is directly or indirectly controlled by Notch signaling in the specification of cell division and/or cell death.

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Now the formal stuff…

Chapter 2: Shelagh Campbell participated in scoring the mutagenesis crosses. Simon Rabinovitch sequenced genomic DNA and identified molecular lesions in the *Dwee1*^{ES1} and *Dwee1*^{ES2} mutants. Christine Walker sequenced the *Dwee*^{DS1} allele. Simon Rabinovitch synthesized the genomic *Dwee1* rescue construct. Shelagh Campbell and Ellen Homola generated transgenic flies. Thanks also to the Berkeley Drosophila Genome Project for the sequence of the genomic clone DS01321. This project began in the laboratory of Pat O'Farrell, and owes much to his advice and guidance. Shelagh Campbell wrote the bulk of this manuscript.

Chapter 3: Shelagh Campbell and Simon Rabinovitch cloned the *Dmyt1* gene. Simon Rabinovitch synthesized the *UAS-Dwee1* and *UAS-Dmyt1* constructs. Zhigang Jin performed the phosphohistone staining in Figure 3-3. Christine Walker assembled the *UASp-Dwee1* clone and assisted with embryo injections. Bruce Hay furnished the GMR plasmid. Gary Ritzel gave advice on synthesizing the GMR clones. Veronica Rodrigues provided the *sd-Gal4* stock. Barbara Thomas sent the *GMR-rux* stock. Jörg Großhans sent the *UAS-trbl* stock, and Exelixis provided the *p53-pExP-glass* stock.

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PREFACE

This thesis is an examination of cell cycle control by the *wee1* kinase of *Drosophila*. The thesis is organized as follows: A current review of cell cycle control is provided in Chapter 1, with emphasis on the function and regulation of Wee1 kinases. My work on *Drosophila wee1* (*Dwee1*) is presented in chapters 2 and 3, and these studies have been published:

Chapter 2: Price, D., S. Rabinovitch, P.H. O'Farrell, and S.D. Campbell. 2000. Drosophila wee1 has an essential role in the nuclear divisions of early embryogenesis. *Genetics* **155**: 159-66.

Chapter 3: Price, D.M., Z. Jin, S. Rabinovitch, and S.D. Campbell. 2002. Ectopic expression of the Drosophila cdk1 inhibitory kinases, wee1 and myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development. *Genetics* **161**: 721-31.

My most recent work with the *Drosophila* Wee1-like kinases is presented in chapter 4. Finally, an overview of my research with respect to cell cycle control is provided in Chapter 5.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

APC/C: anaphase-promoting complex or cyclosome. A large multisubunit protein complex which degrades proteins tagged with the small ubiquitin moiety. The specificity of this degradation is determined by adaptor subunits that confer differential binding affinities to particular substrate molecules. The APC/C participates in mitotic progression by degrading cell cycle regulators, most notably the mitotic cyclins. These proteolytic events are essential for completion of mitosis and cytokinesis.

Balancer chromosome: A marked chromosome containing multiple inversions. These inversions prevent crossing over with its homologue, and can therefore be used to maintain a mutation-bearing chromosome of interest in an intact state.

***cdc* mutants**: For "cell division cycle". A series of temperature sensitive mutants isolated in both budding and fission yeasts which at restrictive temperature disrupt the function of critical factors required for cell cycle progression. The isolation of *cdc* mutants formed the cornerstone of genetic studies of cell cycle control. It should be noted that the numbering of *cdc* mutants is not complementary between budding and fission yeast, so a common *cdc* designation (e.g., *cdc25*) does imply any commonality of gene structure or function.

correspondent

Cdk: cyclin dependent kinase. Associated with a cyclin regulatory subunit, these protein kinases are encoded by a single gene in the yeasts, but have diverged into families in multicellular eukaryotes. These specialized isoforms work in conjunction with specific cyclin partners to drive cycle transitions. Cdks are a major point of regulation in the cell cycle, both by checkpoints as well as serum growth factors.

CKI: Cdk inhibitors. A diverse group of proteins which inhibit the activity of cell cycle stage-specific Cdks by direct binding.

CyO: A dominantly-marked second chromosome balancer.

DNA: deoxyribonucleic acid. The molecule in which genetic information is stored.

DSB: double strand break. A gap in both strands of a DNA molecule. This can occur spontaneously, but is often induced by recombination enzymes or ionizing radiation.

EGF: epidermal growth factor. A family of extracellular ligands that activate a signaling cascade downstream of the EGF receptor (EGFR). This can induce a myriad of cellular responses depending on the identity of the signal-receiving cell. In *Drosophila*, EGFR activating ligands are encoded by *gurken*, *spitz*, and *vein*. An inhibitory EGFR ligand is encoded by *argos*.

EGFR: A family of receptors that bind to extracellular ligands of the EGF class. In *Drosophila*, the EGFR is encoded by a single gene.

EMS: ethyl methanesulfonate. A DNA alkylating agent. A potent mutagen commonly used in genetic screens.

G1 Phase: The first "gap" phase in the cell cycle. The interval following mitosis but preceding S Phase.

G2 Phase: The second "gap" phase in the cell cycle. The interval following S phase, but preceding mitosis.

Gal4: A *P*-element-based "enhancer-trap" vector which drives *S. cerevisiae* Gal4 protein from a weak promoter in the expression domain of the gene next to which it inserts. Combining a tissue-specific Gal4 insertion with a UAS insertion results in the UAS-transgene being ectopically expressed in the domain of Gal4.

GMR: glass multimer reporter. A *P*-element-based expression vector. The GMR plasmid contains a multimer of the Glass transcription factor binding sequence. This drives transgene expression posterior to the morphogenetic furrow (MF) in the larval imaginal eye disc.

HU: Hydroxyurea. An inhibitor of DNA replication which acts by interfering with ribonucleotide reductase, thereby depleting cellular pools of deoxyribonucleotide triphosphates. HU is commonly used to assay DNA replication checkpoint functions.

MF: morphogenetic furrow. Refers to a constricted region that passes across the larval imaginal eye disc late in the third larval instar (starting at the posterior end and progressing anteriorly). The MF marks the dynamic progression of differentiation among the cells that will comprise the ommatidia which make up the compound eye of the adult fly.

MPF: Maturation Promoting Factor (or M Phase-Promoting Factor). The mitosis/meiosis-promoting cyclin-dependent kinase complex consisting of a protein kinase (Cdk1 or $p34^{cdc2}$) and a cyclin regulatory subunit.

***P*-element:** A transposable element common to Drosophilids. This element may be used as a mutagen to disrupt genes or their regulatory sequences, or in a modified form, as a transformation vector which can carry foreign or modified DNA sequences for expression in the fly.

PCR: polymerase chain reaction. A procedure for exponentially amplifying a DNA sequence of interest. Specific DNA primers which flank a region of interest are used to prime multiple rounds of DNA synthesis using a heat-resistant polymerase under a repeated cycle regimen of cool (annealing) warm (extension) and hot (denaturing).

S Phase: The period in the cell cycle during which DNA is replicated.

SEM: scanning electron microscopy.

SMW: second mitotic wave. Posterior to the MF in the larval imaginal eye disc, cells in the ommatidial preclusters signal to the surrounding undifferentiated cells via the secreted EGF ligand, Spitz. This induces them to divide, and protects them from undergoing apoptosis. The SMW is required to generate a pool of cells sufficient for recruitment into the ommatidial preclusters.

TEM: transmission electron microscopy.

***TM6B*:** A dominantly-marked balancer of the third chromosome.

UAS: upstream activating sequence. *P*-element based expression vectors containing a transgene downstream of the UAS site which are part of the two-component Gal4/UAS expression system commonly used in *Drosophila*. Gal4 protein expression can "drive" expression of the transgene by binding to the UAS site and stimulating the basal transcriptional machinery.

1 Introduction

1.1 The cell cycle

Events in the life of a eukaryotic cell must proceed in a defined order. Inadvertent deviation from this order–such as initiating mitosis before completing DNA replication–engenders dire consequences. In a multicellular organism this may portend genomic instability, rendering a cell susceptible to malignant transformation (Hartwell 1992), or lead to developmental impairment. The order and orchestration of these episodes are collectively known as the cell cycle. Eukaryotic cells have evolved elaborate mechanisms (called 'checkpoints') to ensure that the order of events in the cell cycle program is rigorously monitored and controlled. Mutations disrupting the function of these checkpoint controls in humans result in genetic disease, including predisposition to cancer and abnormal development (Malkin *et al.* 1990; Srivastava *et al.* 1990; Castilla *et al.* 1994; Friedman *et al.* 1994; Futreal *et al.* 1994; Miki *et al.* 1994; Simard *et al.* 1994; Savitsky *et al.* 1995; Bell *et al.* 1999; Meijers-Heijboer *et al.* 2002).

Early studies of the cell cycle were largely descriptive, and focused on the timing of alternation between the two most immediately recognizable cell cycle stages, interphase and mitosis (M phase). Later studies subdivided interphase further into the G1, S and G2 phases. S phase is defined as the period in which a cell replicates its genome. This may be perceived by incorporation of radiolabeled nucleotides or nucleotide analogues. G1 by default is the gap between the end of mitosis and the start of the next S phase. G2, similarly, is the gap between the end of S phase and the start of the succeeding mitosis.

Insight into the mechanisms of cell cycle control effectively began with the cell fusion experiments of Rao and Johnson. These involved fusing mammalian tissue culture cells at different stages in the cell cycle and observing the ensuing consequences for the respective nuclei. These investigators noted that if a cell in S phase is fused with a cell in G1, the nucleus of the G1 cell is immediately induced to enter S phase. When a cell in S phase is fused with a cell in G2, the nucleus of the G2 cell is resistant to S phase induction. This indicates that: 1) there are diffusible factor(s) in the cytoplasm of an S-phase cell that can induce S phase; 2) some factor(s) specifies a block to re-replication in a G2 cell and 3) this block is somehow removed following mitosis (Rao and Johnson 1970). A cell in mitosis fused with a cell at any other point in the cell cycle induces the nucleus of that cell to enter mitosis immediately (Johnson and Rao 1970). The background to the field of cell cycle study is reviewed extensively elsewhere (Mitchison 1971; Murray and Hunt 1993).

1.2 Mechanisms of cell cycle control

While the cell fusion experiments of Rao and Johnson suggested a hierarchical machinery controlling the cell cycle, experiments were underway that would identify the physical components behind this phenomenology. Early dissections of cell cycle control took place in seemingly disparate spheres of study: through biochemical analysis of oocyte maturation, and mutational studies in the yeasts.

1.2.1 Oocyte maturation and the search for MPF

Masui and others utilized the large amphibian oocyte. Amphibian oocytes remain arrested in prophase of meiosis I for several months as they develop prior to laying. Somatic follicular cells expose the oocyte to the steroid hormone progesterone, triggering a signaling cascade that releases the oocyte from its meiosis I arrest (Masui 1967; Baulieu and Schorderet-Slatkine 1983). Following activation by progesterone, the nucleus transits meiosis I and again arrests in metaphase of meiosis II (Smith and Ecker 1971; this process is called 'maturation'), where it remains pending release once more by the influx of calcium during fertilization (Meyerhof and Masui 1977), at which point it completes meiosis and begins embryonic development. Masui and Markert (1971) noted that when an immature oocyte is injected with even a small amount of cytoplasm from a mature egg, maturation of the oocyte is induced. In classic biochemical experiments, researchers began to fractionate extracts of mature egg cytoplasm and re-inject the various sub-fractions–the goal being to isolate a pure protein or group of proteins that conferred this activity (Wasserman and Masui 1976). This elusive entity was termed maturation promoting factor, or MPF. The experimental journey that lead to the discovery of MPF has been reviewed by Masui (Masui 1992; Masui 1996).

1.2.2 Cell division cycle mutants in the yeasts

Hartwell *et al.* (1974) and Nurse *et al.* (1976), studying the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively, isolated a series of temperature-sensitive cell-division-cycle (or *cdc*) mutants. Mutants were selected on the basis of arresting at a particular stage in the cell cycle when cultured at restrictive temperature. Through genetic epistasis analysis of double mutant combinations, these researchers began to flesh out a network of interacting gene products controlling cell cycle transitions (Hartwell 1974; Hayles and Nurse 1992).

Although both are commonly known as yeasts, these two model systems are only distantly related in evolutionary terms (Karlin and Ladunga 1994). This distance is reflected in dramatic differences in lifestyle and mode of cell cycle control. *S. pombe* spends the majority of its life cycle in the haploid state, and therefore maintains only a very brief G1 interval. Its extended G2 phase presumably bespeaks the urgency–particularly acute for a haploid cell–of maintaining a recombinational repair template. *S. cerevisiae* exists mainly as a diploid, and therefore has no such constraints on the length of G1. *S. cerevisiae*, furthermore, manifests essentially no G2 phase: many of the hallmarks of mitosis seen in other organisms initiate during S phase in *S. cerevisiae*. Owing largely to the simple length of these intervals in the respective species, the majority of cell cycle controls, including those responsive to mating cues, nutritional status and DNA damage were initially the focus of study at G1/S in *S. cerevisiae*, and G2/M in *S. pombe*. The control of G1/S by mating factors in *S. cerevisiae* has proven a useful analogue to cellular signaling systems in higher eukaryotes, where growth and entry into the cell cycle are often governed by serum growth factors at this point. Conversely, the mechanism employed by *S. pombe* in controlling the onset of mitosis resembles more closely that which was widely adopted in multicellular eukaryotes (for a detailed exposition of this theory, see Murray and Hunt 1993). Since my discussion concentrates largely on regulation of passage through the S/M and G2/M transitions, I relate primarily from the *S. pombe* literature, and interject material from the *S. cerevisiae* system only where there are issues of mechanistic conservation/divergence worth noting.

1.3 MPF consists of p34^{cdc2} and cyclin B

Many of the genes involved in cell cycle control are highly conserved throughout evolution. This conservation is so stringent that it frequently extends beyond mere sequence to the level of function. Numerous cell cycle regulatory factors from higher eukaryotes have been cloned on the basis of their ability to complement yeast cells mutant for the homologous gene (Lee and Nurse 1987; Lehner and O'Farrell 1990; Colasanti *et al.* 1991; Lahue *et al.* 1991; Alphey *et al.* 1992; Plon *et al.* 1993; Campbell *et al.* 1995). The first cell cycle gene to be examined in such a way was the *cdc2+* gene of *S. pombe*. The *CDC28* gene of *S. cerevisiae* is the functional equivalent of *cdc2+*, and the two genes are exchangeable in the opposite species (Beach *et al.* 1982). These genes are required for initiating DNA replication as well as mitosis (Nurse and Bissett 1981; Reed and Wittenberg 1990). The biochemical and genetic schools of cell cycle research converged when it was demonstrated that purified MPF includes a Cdc2/Cdc28-like protein (named p34^{cdc2}; Arion *et al.* 1988; Dunphy *et al.* 1988; Gautier *et al.* 1988; Labbe *et al.* 1988). The *cdc2+* gene encodes a universally conserved protein kinase that is essential for mitosis (Nurse 1990).

Another component of MPF is a protein called cyclin B (Draetta *et al.* 1989; Labbe *et al.* 1989; Gautier *et al.* 1990). Cyclin proteins were first identified in cycling sea urchin embryo extracts treated with radiolabeled amino acid (Evans *et al.* 1983). Most cellular proteins accumulate label in a linear fashion over time, but the cyclins accumulate label and are then degraded at intervals that cycle with the periodicity of a single cell cycle. The cyclin proteins serve as vital regulatory subunits controlling the activity of p34^{cdc2} (Hagan *et al.* 1988; Booher *et al.* 1989; Draetta *et al.* 1989; Labbe *et al.* 1989; Meijer *et al.* 1989; Moreno *et al.* 1989; Murray and Kirschner 1989a; Pines and Hunter 1989). In many early metazoan embryos the activity of p34^{cdc2} is controlled solely by the periodic accumulation and degradation of cyclin (Murray and Kirschner 1989a; Murray *et al.* 1989).

Upon activation, p34^{cdc2}/cyclin B phosphorylates (either directly or indirectly) a wide range of proteins (Newport and Kirschner 1984; Karsenti *et al.* 1987; Lohka *et al.* 1987). This is thought to drive the cellular events characteristic of mitosis (nuclear envelope breakdown, spindle assembly, chromosome condensation etc; Miake-Lye *et al.* 1983; Newport and Kirschner 1984; Miake-Lye and Kirschner 1985). At the conclusion of mitosis ubiquitination targets the cyclin subunit of the complex for degradation by the anaphase-promoting complex (APC/C) or cyclosome (Felix *et al.* 1990; Glotzer *et al.* 1991). Failure to degrade cyclins blocks exit from mitosis (Murray *et al.* 1989).

In the yeasts the Cdc2/Cdc28 proteins drive cell cycle progression in association with a variety of cell cycle stage-specific cyclin family isoforms (Fisher and Nurse 1995; Andrews and Measday 1998). In higher eukaryotes the *cdc2+/CDC28*-like genes have diverged into families, with multiple variant isoforms (called cyclin-dependent kinases, or Cdks) utilized for driving particular cell cycle transitions in association with specialized cyclin family members (reviewed by Pines 1993). To illustrate this point, Table 1-1 contains a brief summary comparison of Cdk/cyclin counterparts acting at cell cycle transition points in vertebrates and budding yeast.

In a recent move toward a unified nomenclature, mitotic $p34^{cdc2}$-like kinases are designated as Cdk1, although the now archaic use of Cdc2 to refer to this type of kinase in any organism remains quite prevalent. I hereafter use the generally accepted Cdk1 in generic reference to this protein, excepting specific references to its named homologues in other organisms, such as *S. pombe* where I revert to the original Cdc2, or in *S. cerevisiae* with Cdc28.

1.4 The $p34^{cdc2}$/cyclin B complex is controlled by phosphorylation

Two proteins, Wee1 and Cdc25, are of particular importance to the study of G2/M progression. Prominent among the extensive studies of cell cycle control in *S. pombe* by Nurse and colleagues, was the identification of a mutant defective in cell-size control (Nurse 1975). In *wee1+* cells, cell size at mitosis is approximately constant under logarithmic growth, but is reduced significantly upon nutrient limitation; the opposite response is seen when cells are shifted from nutrient-poor to nutrient-rich medium (Fantes and Nurse 1977). *wee1* cells divide at a size approximately 50% that of wild type (hence the name, wee), and are incapable of responding to shifts in nutrient sources (Fantes and Nurse 1978). *wee1* cells display a greatly shortened G2 phase, with a compensatory increase in the length of G1 (Nurse 1975).

While *wee1* cells are small due to entering mitosis prematurely, $cdc2^{ts}$ and $cdc25^{ts}$ cells fail to enter mitosis at the restrictive temperature; instead continuing to grow with a G2 DNA content. Rare mutations in the *cdc2+* gene yielding a 'wee-like' phenotype are also found, however whereas mutations in *wee1+* are typically recessive, these mutations in *cdc2+* (*cdc2-w*) are generally dominant (Nurse and Thuriaux 1980). Genetic interaction analyses revealed that the *wee1+* gene product probably antagonizes the product of the *cdc25+* gene. The combination of both *wee1* and *cdc25* mutations produces cells that divide at close to wild type size even at restrictive

CYCLIN-CDK	VERTEBRATES		BUDDING YEAST	
COMPLEX	CYCLIN	CDK PARTNER	CYCLIN	CDK PARTNER
G1-Cdk	Cyclin D1, 2, 3	Cdk4, Cdk6	Cln3	Cdk1
G1/S-Cdk	Cyclin E	Cdk2	Cln1, 2	Cdk1
S-Cdk	Cyclin A	Cdk2	Clb5,6	Cdk1
M-Cdk	Cyclin B	Cdk1	Clb1, 2, 3, 4	Cdk1

Table 1-1

A summary of Cdk/cyclin counterparts driving cell cycle transitions in vertebrates and budding yeast. Adapted from Alberts *et al.* (2002). Reprinted with permission of the publisher.

temperature. Fantes (1981) went on to cleverly posit that the *wee1+* and *cdc25+* gene products function antagonistically in regulating the activity of the *cdc2+* gene product, with Cdc25 assuming an activating role and Wee1 inhibitory. This conclusion was based on a screen for suppressors of *cdc25*: the great majority were recessive alleles of *wee1*, while rare dominant *wee* alleles of *cdc2* could also suppress the *cdc25* block.

Russell and Nurse later added significant validity to this model in a series of landmark experiments. Both *cdc25+* and *wee1+* were cloned by complementation (Russell and Nurse 1986; Russell and Nurse 1987b). Overexpression of each gene mimics loss of the other. Additionally, certain of the dominant *cdc2w* alleles are sensitive to loss of *wee1* but not *cdc25*, and the converse. This argues that the activity of the *cdc2+* gene product is limiting for mitosis, and mutation can render it selectively insensitive to regulation by either Cdc25 or Wee1.

The Wee1 protein has since been shown to act as a protein kinase, inhibiting mitosis by phosphorylating Cdk1 on Y15 (Featherstone and Russell 1991; Parker *et al.* 1992; Parker and Piwnica-Worms 1992; McGowan and Russell 1993). This phosphorylation serves to impede activation of the Cdk1/cyclin B complex. *mik1+*, encoding a closely related kinase with partially redundant function has also been identified in *S. pombe* (Lundgren *et al.* 1991; Lee *et al.* 1994). *mik1* cells have no obvious phenotype, but this mutation is synthetically lethal with *wee1*. Wee1 and Mik1 have overlapping functions, as loss of both proteins causes mitotic catastrophe (Lundgren *et al.* 1991), but each also appears to have subtle unique functions. Wee1 is most obviously required for cell size control at G2, while Mik1 plays a role in the genome integrity checkpoints (see below).

Another Wee1-like kinase has been identified in metazoan organisms; the Myt1 (for membrane-localized tyrosine/threonine-directed) kinase displays a distinct subcellular localization and substrate specificity from Wee1. While Wee1 is usually localized to the nucleus, Myt1 contains a membrane retention signal for the endoplasmic reticulum and Golgi complex (Kornbluth *et al.* 1994; Mueller *et al.* 1995b; Liu *et al.* 1997). Furthermore, their contrasting patterns of localization probably engender non-identity in the sets of Cdk/cyclin substrates for these two kinases. Specialized Cdk/cyclin complexes may exhibit distinct subcellular localization, and this localization is thought to be determined by the identity of the specific cyclin partner (Pines and Hunter 1991; Pines and Hunter 1994; Jackman *et al.* 1995). The choice of cyclin partner may also translate into differences in Cdk activity, substrate-specificity or both (Stiffler *et al.* 1999; Miller and Cross 2000; Draviam *et al.* 2001; Edgington and Futcher 2001; Miller and Cross 2001). Whereas Wee1 phosphorylates Cdk1 exclusively on Y15, Myt1–while demonstrating a preference for T14 *in vitro*–can phosphorylate either residue (Mueller *et al.* 1995b; Booher *et al.* 1997; Liu *et al.* 1997). Myt1 can also bind directly to Cdk1, and overexpression of Myt1 causes an extension of G2 with Cdk1/cyclin B restricted to the cytoplasm (Liu *et al.* 1999; Wells *et al.* 1999). The physiological significance of this mode of regulation remains to be seen.

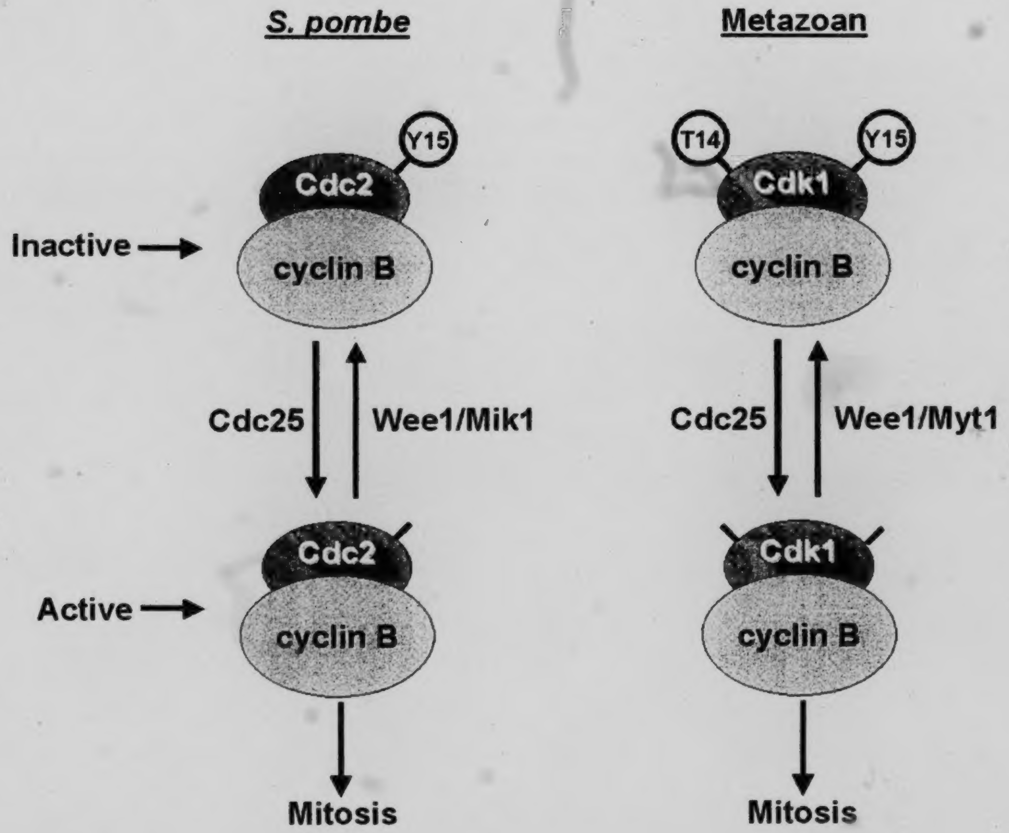
S. pombe
Metazoan
Y15
Cdc2
cyclin B
T14
Y15
Cdk1
cyclin B
Inactive
Cdc25
Wee1/Mik1
Cdc25
Wee1/Myt1
Active
Cdc2
cyclin B
Cdk1
cyclin B
Mitosis
Mitosis

Figure 1-1
The activity of Cdk1-like kinases (and the onset of mitosis) is controlled in part by a balance of Cdk1 phosphorylation/dephosphorylation that is determined by the relative activities of Wee1-like kinases and Cdc25-like phosphatases. Phosphorylation of critical inhibitory residues (Y15 in *S. pombe*; T14 and Y15 in metazoans) prevents Cdk1 activation, delaying mitosis, while removal of these phosphate moieties activates the complex and promotes mitosis.

The Cdc25 protein is a dual-specificity protein phosphatase that acts to promote mitosis by removing the same phosphate moieties affixed by Wee1-like kinases (Gautier *et al.* 1991; Strausfeld *et al.* 1991; Lee *et al.* 1992). Several Cdc25 isoforms exist, and these exhibit substrate preference for activating particular Cdk/cyclin complexes (reviewed by Nilsson and Hoffmann 2000). A cartoon diagram depicting the antagonism of Wee1 and Cdc25 homologues in the control of Cdk1 activity is shown in Figure 1-1.

The Cdc25 phosphatase is activated at the onset of mitosis by a positive feedback loop involving Cdk1. Cdc25 is phosphorylated on multiple sites by Cdk1 and these phosphorylations in turn increase the catalytic activity of Cdc25 for removing inhibitory phosphates from Cdk1 (Hoffmann *et al.* 1993; Izumi and Maller 1993). Coordinately, Wee1 is also phosphorylated by Cdk1, and this decreases its ability to phosphorylate Cdk1 (Honda *et al.* 1995; Mueller *et al.* 1995a). Initiation of this feedback loop appears to require the activity of the Polo-like family of kinases. Polo kinases are named after the *Drosophila* mutant in which this gene is disrupted (Sunkel and Glover 1988; Llamazares *et al.* 1991), and are widely conserved. Polo-like kinases phosphorylate Cdc25 homologues to initiate mitosis (Kumagai and Dunphy 1996; Abrieu *et al.* 1998; Karaiskou *et al.* 1998; Qian *et al.* 1998; Qian *et al.* 2001). As might be expected given their role in activating Cdc25, Polo-like kinases have also been shown to participate in the phospho-inactivation of Wee1 homologues (Abrieu *et al.* 1998; Bartholomew *et al.* 2001; Kang *et al.* 2002). Polo-like kinases are also required for exiting mitosis (Descombes and Nigg 1998) and cytokinesis (Carmena *et al.* 1998; Song and Lee 2001), are targets of the mitotic DNA repair checkpoint (Sanchez *et al.* 1999; Smits *et al.* 2000; van Vugt *et al.* 2001), and are required for proper assembly of mitotic spindles (Sunkel and Glover 1988; Llamazares *et al.* 1991; Ohkura *et al.* 1995; Lane and Nigg 1996; Qian *et al.* 1998).

Cdk1 is subject to regulation by a number of cellular signals, several of which act through modulation of its activity by phosphorylation. In addition to phosphorylation/dephosphorylation by Wee1-like kinases and the Cdc25 phosphatase, Cdk activating kinases (or CAKs) permit activation of Cdc2/cyclin B by phosphorylating threonine 167 in S *pombe*, or an analogous residue on Cdk1 in other species (Ducommun *et al.* 1991; Gould *et al.* 1991).

Finally, a highly divergent class of proteins called cyclin-dependent kinase inhibitors (or CKIs) function to block the activity of the various cell cycle transition-specific Cdks (Harper *et al.* 1993; Serrano *et al.* 1993; Xiong *et al.* 1993; Polyak *et al.* 1994; Toyoshima and Hunter 1994; Foley *et al.* 1999). CKIs achieve this inhibition through binding directly to the Cdk subunit, and can obstruct its binding to cyclins, block access to substrates, influence localization and reduce stability. The expression and stability of CKIs is cell-cycle regulated and is integrated with the other mechanisms for ensuring tight control over Cdk activity.

1.5 Checkpoints control the order of many cell cycle events

It is of critical importance that events proceed in a specified order during the cell cycle. During early studies of the cell cycle, there was much speculation about the mechanisms responsible for enforcing this order. Two models reflected the extremes of opinion on this matter. In the 'dominoes' model, the cell cycle proceeds as a linear series of chemical processes; the completion of early events necessarily precedes later ones, as completed early events furnish chemical substrates required for later events to occur. In the 'clock' model, early and late events in the cell cycle are uncoupled mechanistically, but occur in a defined order due to the action of timing mechanisms, either inherent to the processes themselves, or the result of the action of a 'master' cell cycle timer.

In eukaryotic cells, when timely completion of a cell cycle process is blocked–either by mutation or by exposure to a chemical inhibitor–the cell cycle will arrest at the point of blockage and proceed no further. This is an excellent indication that the clock model is inadequate to explain the order of events in the cell cycle, but remains consistent with the domino model. The demise of both models came through identification of mutants that fail to arrest in response to blocked cell cycle events.

Yeast cells will normally arrest if cell cycle events are blocked. This includes either obstruction of DNA replication or DNA damage. Additionally, cells arrest in metaphase of mitosis if microtubule assembly is disrupted. Extensive genetic screens in both yeasts have identified mutants which fail to arrest in response to each of these treatments. These mutants define what have become known as checkpoint controls of the cell cycle. Hartwell and Weinert (1989) elucidated the classic example.

RAD9 had previously been identified as an *S. cerevisiae* gene required for preventing mitosis in the presence of DNA strand breaks (Weinert and Hartwell 1988). The *CDC9* gene encodes the DNA ligase enzyme, and the *cdc9* mutant normally arrests in metaphase at a restrictive temperature. When combined with *rad9*, however, the double mutant proceeds through mitosis with unligated Okazaki fragments, producing fragmented chromosomes and dead cells (Schiestl *et al.* 1989). This experiment proved conclusively that (at least in the case of the dependency relationship between DNA repair and mitosis) there is an active checkpoint mechanism that *senses* the completion of one cell cycle event (repair of DNA strand breaks) and *permits* the next event (exit from mitosis) to occur. These models are discussed in detail by Murray and Kirschner (1989b) and numerous others.

1.5.1The S/M and G2/M checkpoints act through phosphorylation of Cdk1

Several large-scale screens have been conducted in *S. pombe* to identify mutations that confer sensitivity to inhibitors of DNA replication and ionizing radiation. These screens produced a set of six genes required for both the

DNA replication (S/M) and repair (G2/M) checkpoint responses (al-Khodairy and Carr 1992; Enoch *et al.* 1992; Rowley *et al.* 1992). These so-called 'checkpoint *rad*' genes are thought to interact at the most proximal level to the physical structures of stalled replication forks or various forms of DNA damage. This group of proteins relays the signal via a protein kinase cascade that ultimately reaches Cdk1.

The genes represented by each of these checkpoint *rad* mutations have since been cloned, and conserved homologues have been identified and characterized in a variety of organisms (Hari *et al.* 1995; Savitsky *et al.* 1995; Barlow *et al.* 1996; Bentley *et al.* 1996; Bluyssen *et al.* 1998; Dean *et al.* 1998; Freire *et al.* 1998; Parker *et al.* 1998; Bluyssen *et al.* 1999; Bessho and Sancar 2000; de Klein *et al.* 2000; Komatsu *et al.* 2000; Weiss *et al.* 2000; Cortez *et al.* 2001). Of particular importance to the current discussion are the homologues of *rad3+*, which have been shown to play important roles in multiple checkpoint mechanisms. These include the *S. cerevisiae MEC1* and *TEL1* genes, the *Drosophila mei-41* gene, and the vertebrate *ATM* and *ATR*-like genes (Bentley *et al.* 1996).

Inhibitory phosphorylation of Cdc2 is essential for the DNA damage and replication checkpoints in *S. pombe* (Enoch and Nurse 1990; Rhind *et al.* 1997; Rhind and Russell 1998b). Replacement of Y15 with the non-phosphorylatable F drives cells into premature mitosis, and abrogates both checkpoint responses (Enoch *et al.* 1991; Rhind *et al.* 1997; Rhind and Russell 1998b). The *S. pombe chk1+* gene encodes a protein kinase that is required for delaying mitosis in response to ionizing and ultraviolet radiation (Walworth *et al.* 1993). *chk1* cells show only marginal sensitivity to inhibitors of DNA replication such as hydroxyurea (HU), and as such, Chk1 seems to play a minor role in the normal DNA replication checkpoint response in *S. pombe*. Overexpression of *chk1+* causes delayed mitosis and elongated cells, similar to the effect of weak alleles of *cdc25*. Overexpression of *chk1+* can partially rescue the radiation sensitivity of the series of checkpoint *rad* mutants, and therefore is thought to act downstream of these proteins (Walworth and Bernards 1996).

Chk1 is phosphorylated in response to ionizing radiation; this phosphorylation appears to be auto-catalyzed and is contingent on checkpoint *rad* gene function. Chk1 has been shown to phosphorylate a number of sites on Cdc25 *in vivo* and *in vitro* in a variety of systems (Furnari *et al.* 1997; Sanchez *et al.* 1997). This phosphorylation occurs in response to ionizing radiation, and either directly or indirectly blocks activation of the Cdk1/cyclin B complex. Phosphorylation of Cdc25 by Chk1 creates a binding site for interaction of Cdc25 with the 14-3-3 class of proteins (Peng *et al.* 1997). This interaction serves to target Cdc25 to the cytoplasm, as a nuclear export signal on 14-3-3 proteins causes them to be constitutively exported from the nucleus (Lopez-Girona *et al.* 1999). In *S. pombe* this would suffice to prevent access to its substrate (the Cdk1/cyclin B complex), but in other systems MPF is localized cytoplasmically as well, and nuclear/cytoplasmic compartmentalization appears insufficient to account for the checkpoint activation. In fact a recent study concludes that nuclear exclusion of Cdc25 is not required for checkpoint function even in *S. pombe* (Lopez-Girona, Kanoh and Russell, 2001). There is evidence from *Xenopus*, *S. pombe* and human cells pointing to a direct inhibitory effect of phosphorylation by Chk1 and 14-3-3 binding on Cdc25 activity (Kumagai *et al.* 1998; Furnari *et al.* 1999; Morris *et al.* 2000), but the matter appears far from resolved.

The Cds1 kinase was also first identified in *S. pombe*, and is required for recovery from blocked DNA replication (Murakami and Okayama 1995). Cds1 functions redundantly with Chk1 to enforce the DNA replication checkpoint and is required for responding to DNA damage induced during S phase (Boddy *et al.* 1998; Lindsay *et al.* 1998; Martinho *et al.* 1998; Rhind and Russell 1998a; Zeng *et al.* 1998; Brondello *et al.* 1999; Furnari *et al.* 1999). Chk1 is only induced by HU in *cds1* cells (Brondello *et al.* 1999).

There has been some controversy in the literature over the relative contributions of Wee1 vs. Cdc25 to the genome integrity checkpoint signals. The regulation of Cdc25 in response to these checkpoints has been comparatively easy to define, while roles for Wee1 have proven enigmatic. Wee1 is likewise phosphorylated in response to DNA damage and Chk1 overexpression, and is phosphorylated *in vitro* by activated Chk1 (O'Connell *et al.* 1997). Wee1 levels increase in response to DNA damage (Raleigh and O'Connell 2000). Wee1 also interacts with 14-3-3 proteins in response to phosphorylation by Chk1, and this has been shown to increase both its stability and kinase activity (Wang *et al.* 2000; Lee *et al.* 2001a). However other studies in *S. pombe* have shown that the DNA damage checkpoint is independent of Wee1 function (Furnari *et al.* 1997; Rhind *et al.* 1997; Rhind and Russell 2001). The Mik1 kinase is stabilized in response to DNA damage through a mechanism requiring Chk1 (Baber-Furnari *et al.* 2000; Christensen *et al.* 2000), and is required for enforcing this checkpoint (Rhind and Russell 2001).

Treatment of *S. pombe* cells with HU causes Cds1-dependent phosphorylation of Wee1, and an increase in the level of Mik1 protein (Boddy *et al.* 1998; Christensen *et al.* 2000). But again other studies indicate that (at least in *S. pombe*) Mik1 is required for the DNA replication checkpoint while Wee1 is inessential (Baber-Furnari *et al.* 2000; Rhind and Russell 2001). In *Xenopus*, mitosis is dependent on ubiquitin-mediated degradation of Wee1 after S phase, and the DNA replication checkpoint acts, at least in part, through maintaining Wee1 stability (Michael and Newport 1998). As was shown for Chk1 in the DNA damage checkpoint response, Cds1 can also phosphorylate Cdc25 and promote its interaction with 14-3-3 proteins (Boddy *et al.* 1998; Zeng *et al.* 1998; Furnari *et al.* 1999).

While Chk1 seems to respond exclusively to DNA damage in *S. pombe*, and Cds1 to blocked DNA replication, work in other systems indicates that this strict division of labour does not persist throughout evolution. The response specificity of Chk1 and Cds1 in cycling *Xenopus* egg extracts is opposite from that observed in *S. pombe* (Guo and Dunphy 2000). Chk1 and Cds1 homologues have also been identified in humans (CHK1 and

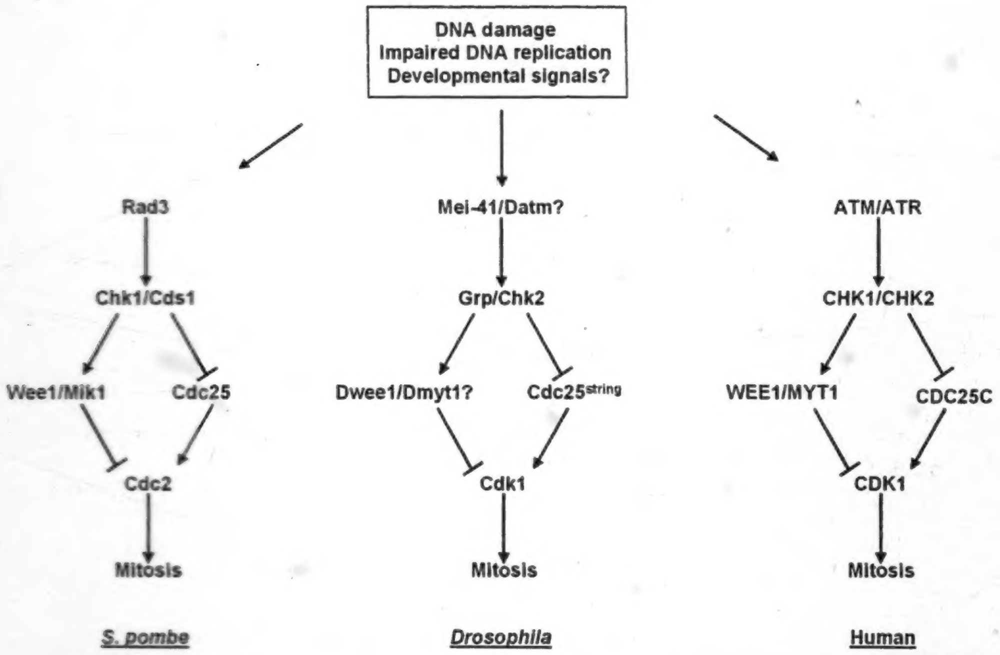
DNA damage
Impaired DNA replication
Developmental signals?
Rad3
Chk1/Cds1
Wee1/Mik1
Cdc25
Cdc2
Mitosis
S. pombe
Mei-41/Datm?
Grp/Chk2
Dwee1/Dmyt1?
Cdc25string
Cdk1
Mitosis
Drosophila
ATM/ATR
CHK1/CHK2
WEE1/MYT1
CDC25C
CDK1
Mitosis
Human

Figure 1-2
A conserved protein phosphorylation cascade links checkpoint signals to the control of mitosis. Pointed arrows indicate stimulatory interactions, while blunt ends represent inhibitory signals. For simplicity, the ATM/ATR kinase signal streams have been amalgamated. Question marks indicate *Drosophila* homologues for which little is known.

CDS1/CHK2; Sanchez *et al.* 1997; Matsuoka *et al.* 1998; Brown *et al.* 1999). These proteins both appear to be induced to phosphorylate CDC25C in response to DNA damage as well as blocked DNA replication.

A variety of other checkpoint mechanisms exist in metazoans, and several of them are also targets of CHK1 and CDS1. CHK1 and CDS1/CHK2 both phosphorylate the DNA damage response transcription factor p53 at DNA damage-inducible sites (Shieh *et al.* 2000). Ionizing radiation induces ATM-dependent phosphorylation of p53 by CDS1/CHK2 (Chehab *et al.* 2000; Hirao *et al.* 2000). This phosphorylation dramatically increases p53 stability. CDS1/CHK2 has also been shown to activate the DNA damage response protein BRCA1 by phosphorylation following DNA damage (Lee *et al.* 2000). Activated BRCA1 in turn activates CHK1 (Yarden *et al.* 2002). The broad outline of the conserved pathway of factors controlling entry into mitosis is summarized in Figure 1-2.

1.5.1.1 In budding yeast, the genome integrity checkpoints do not function through inhibitory phosphorylation of Cdk1

Contrary to the situation in *S. pombe* and other organisms, inhibitory phosphorylation of Cdc28 on Y19 (the analogous residue to Y15 in *S. pombe*) plays no role in the mitotic DNA replication or damage checkpoints in *S. cerevisiae*. Mutation of this site to a non-phosphorylatable residue (Y19F) produces no perceptible advancement of mitosis following DNA damage or blocked replication (Amon *et al.* 1992; Sorger and Murray 1992).

Again in contrast to what is seen in *S. pombe*, control of cell division in response to the DNA damage checkpoint in *S. cerevisiae* centers on controlling exit from, rather than entry into mitosis. The upstream signaling components appear to be conserved from more typical checkpoints targeting Cdk1 inhibitory phosphorylation, but the target of this signal has changed. Exit from mitosis in budding yeast occurs in a step-wise fashion, with specialized APC/C-adaptor protein isoforms responsible for degrading chromatid cohesion factors and mitotic cyclins.

The securin anaphase inhibitor Pds1 prevents sister chromatid separation by stabilizing the Scc1 cohesin (Ciosk *et al.* 1998; Uhlmann *et al.* 1999) which links sister chromatids (Michaelis *et al.* 1997). *pds1* cells are compromised for the spindle assembly and mitotic DNA damage checkpoints (Yamamoto *et al.* 1996). Pds1 is stabilized in response to DNA damage via a pathway involving Mec1 (homologous to *S. pombe* Rad3) and a Chk1 homologue (Cohen-Fix and Koshland 1997; Sanchez *et al.* 1999). Pds1 is phosphorylated directly by Chk1, and this prevents its degradation by APC^{Cdc20} (Wang *et al.* 2001). In keeping with its multiplicity of roles throughout the cell cycle, a Polo-like kinase, Cdc5, plays a redundant role with Pds1 in controlling Scc1 degradation and sister chromatid separation (Alexandru *et al.* 2001).

Pds1 also plays a role in mitotic exit–ensuring the stability of mitotic cyclins by preventing activation of the APC^{Cdh1} complex which targets them for degradation (Cohen-Fix and Koshland 1999; Shirayama *et al.* 1999;

Tinker-Kulberg and Morgan 1999; Schwab *et al.* 2001a). Another partially redundant pathway controlling mitotic exit involves Mec1 and Rad53 (homologous to Cds1 of *S. pombe*) and inhibits the Polo-like protein, Cdc5, in response to DNA damage (Sanchez *et al.* 1999). This blocks Cdc5-induced activation of APC^{Cdh1}in degrading mitotic cyclins (Hu *et al.* 2001; Lee *et al.* 2001b).

1.6 Cell cycle control in the early *Drosophila* embryo

As a model organism, *Drosophila* lies between the two extremes presented by the *Xenopus* and yeast systems: being one of the more genetically tractable metazoan organisms, it lends itself readily to mutational analysis of cell cycle control in development. The groundwork for cell cycle study in *Drosophila* was laid by Foe and Alberts (1983) and Foe (1989), who enumerated the morphological changes traversed in the development of the early embryo. This provided an invaluable frame of reference for future researchers, particularly with respect to mutational and inhibitor studies. The effects of a given mutation or inhibitor could be compared to the standard established by these works.

Subsequent to fertilization the *Drosophila* embryo undergoes 13 rapid maternally driven mitotic cycles. These occur metasynchronously and without gap phases, in a common cytoplasm, or syncytium. The earliest cycles proceed extremely rapidly (total cycle time ~8 minutes). The proliferating nuclei occupy the interior of the embryo until cycles 9-10, when the majority migrate outwards to reside at the extreme embryo cortex. A few nuclei remain in the interior of the embryo; these 'yolk nuclei' cease dividing after cycle 11 whereupon they endoreplicate. During cycles 10-13, the division rate of the cortical nuclei progressively slows from the frenetic pace of the early cycles; this is largely due to the increasing length of interphase (cycle 11: 10min; cycle 12: 12 min; cycle 13: 21min). It is during this slowing of nuclear cycles that zygotic gene transcription is first initiated (Edgar *et al.* 1986; Edgar and Schubiger 1986).

The lengthening of interphase seen in the late syncytial cycles is a function of the increasing ratio of nuclei to cytoplasm (Edgar *et al.* 1986); this has also been postulated to represent the action of a maternally specified DNA replication checkpoint (Sibon *et al.* 1997). With analogy to early *Xenopus* development (Dasso and Newport 1990), it has been proposed that completion of S phase in the earliest cycles (1-9) requires less time than is needed for the mitotic machinery–presumably driven by cyclin synthesis and degradation–to cycle. Maternally supplied DNA replication factors are progressively titrated by the exponentially dividing population of nuclei, to the extent that they become limiting. The interval of time required to complete S phase now exceeds that of the mitotic oscillator. A checkpoint mechanism is therefore essential to delay mitosis until S phase is completed.

An extended interphase follows mitosis 13; this is comprised of a 40 minute S phase followed by the first embryonic G2 phase. It is during this extended interphase that the embryo passes control of its cell division and patterning programs from maternal to zygotic gene products (Edgar *et al.* 1986; Edgar and Schubiger 1986; Edgar and Datar 1996). Additionally, it is during this interphase that cell membranes form around the cortical nuclei, partitioning the syncytial blastoderm into a cellular blastoderm (Schweisguth *et al.* 1991; Schejter and Wieschaus 1993). Many maternal messages are degraded during interphase 14, and zygotic gene transcription commences in earnest. This is known as the midblastula, or maternal/zygotic transition (Foe *et al.* 1993). Concomitant with the onset of gastrulation, and in contrast with the metasynchronous waves of mitosis seen in the syncytial cycles, pulses of zygotic *cdc25^{string}* transcription drive spatially and temporally distinct groups of G2 cells into mitosis in what are termed the mitotic domains (Edgar and O'Farrell 1989; Edgar and O'Farrell 1990). The descendants of cells within a given mitotic domain go on to occupy a common position in the fate map of the organism later in development (Foe 1989).

1.6.1 Wee1 kinases in *Drosophila*

Campbell *et al.* (1995) cloned a *wee1* homologue from *Drosophila* (*Dwee1*) by complementing the lethal mitotic catastrophe of the *S. pombe wee1 mik1* double mutant. *Dwee1* shows significant conservation with its *Xenopus* and human homologues. The gene was localized cytologically by *in situ* hybridization to salivary gland polytene chromosomes. A *P*-element excision-derived deletion served as the starting material for the present study. A mutagenesis screen was conducted to identify mutations in *Dwee1* (Price *et al.* 2000). We hoped that this would allow us to assess the function of this inhibitory kinase, and the role of Cdk1 inhibitory phosphorylation in the development of a metazoan organism.

Lengthening of interphase in the late syncytial cycles is obstructed by maternal-effect mutations in the *grapes* (*grp*–homologue of *chk1+;* Fogarty *et al.* 1997; Sibon *et al.* 1997), *mei-41* (homologue of *rad3+;* Hari *et al.* 1995; Sibon *et al.* 1999), and *mus304* (homologue of *rad26+*; Brodsky *et al.* 2000; Cortez *et al.* 2001) genes. Embryos from maternal mutants for *Dwee1* exhibit the same phenomenon (Price *et al.* 2000; this study). Embryos produced by mothers homozygous for any of these mutations develop relatively normally until maternal cycles 12 and 13 whereupon they enter mitosis prematurely, leading to the appearance of chromosomal bridges as nuclei endeavor to segregate ostensibly underreplicated chromosomes. Eventually the nuclei coalesce into large aggregates and these embryos fail to complete syncytial development. These mutations ablate known homologues of DNA replication/repair checkpoint pathway genes. It therefore makes intuitive sense that removal of these genes could abolish a DNA replication checkpoint that is responsible for lengthening the late syncytial interphases.

While immediately appealing, there is very little in the way of physical evidence to support the model of a syncytial DNA replication checkpoint acting via inhibitory phosphorylation of Cdk1. Inhibitory Y15 phosphorylation of Cdk1 is barely detectable at this stage (Edgar *et al.* 1994), and is not diminished in *Dwee1*-derived embryos vs. wild type (E. Homola and S.

Campbell, personal communication). The observed phosphorylation may be catalyzed by another *wee1*-like kinase (discussed below).

Surprisingly, loss of *Dwee1* in homozygous or hemizygous mutants appears to have little effect on zygotic development. Apart from sensitivity to HU (Price *et al.* 2000) and caffeine (Radcliffe *et al.* 2002, in press), loss of zygotic *Dwee1* confers only a marginal detriment on viability. Since pulses of Cdc25^{string} drive the patterned mitoses in the cellular blastoderm of the early embryo (Edgar and O'Farrell 1989), these cells are presumably held in G2 by Wee1-like kinase-catalyzed inhibitory phosphorylation of Cdk1 (the Y15-phosphorylated form is abundant at this stage; Edgar *et al.* 1994). *Drosophila* embryos can withstand ubiquitous heat shock-driven Cdc25^{string} expression, which causes all cells to enter mitosis nearly simultaneously (Edgar and O'Farrell 1990), although defects in gastrulation are observed. Complete zygotic loss of inhibitory Wee1-like kinase function should theoretically produce a similar outcome: cells would enter mitosis as soon as S phase of cycle 14 is completed. Since this effect is not observed in *Dwee1* mutant embryos (Campbell *et al.* 1995), there may be a redundant Wee1-like kinase acting at this stage. A *Drosophila Myt1 (Dmyt1)* kinase homologue has been identified (Cornwell *et al.* 2002; Price *et al.* 2002), and Dmyt1 may perform essential zygotic *wee1* kinase functions. Alternatively, the two kinases may function redundantly, and developmental defects would only manifest upon simultaneous loss of both proteins. Mutants for *Dmyt1* have been isolated (Z. Jin, personal communication), so it is now possible to directly test these options.

1.6.2 Regulation of Wee1 kinases

The first example of a specific regulator of a Wee1 kinase came in *S. pombe*, with the discovery of the *nim1+/cdr1+* gene. Two groups discovered *nim1+* independently; one screening for mutants defective in their response to nutrient limitation, the other in a screen for multicopy suppressors of *cdc25* (Russell and Nurse 1987a; Young and Fantes 1987). *nim1+* encodes a widely conserved kinase, and the Nim1 protein has been shown to promote mitosis by directly phosphorylating and thereby inhibiting the activity of the Wee1 kinase (Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993). However the signals upstream of Nim1 remain unclear.

Somewhat ironically–given its early dismissal as unimportant–the most clearly defined paradigm for regulation of a Wee1 kinase exists in budding yeast. While inessential for enforcing the mitotic DNA replication and repair checkpoints, Swe1-mediated phosphorylation of Cdc28 is required for mitotic control in response to a number of signals.

When nitrogenous nutrients become limiting, an unknown sensor mechanism activates a MAP kinase-signaling pathway, which functions at least in part through the *nim1*-related kinases Elm1 and Hsl1 to increase the stability and/or activity of Swe1 (Ahn *et al.* 1999; Edgington *et al.* 1999; La Valle and Wittenberg 2001). Stabilization of Swe1 inhibits Cdc28/Clb activity, which prevents activation of the isotropic mode of growth (growth which occurs in all areas of the bud at once, and produces a spherical daughter cell). Continued apical growth (which occurs at the distal end of the bud only) produces long rod-like or filamentous arrangements of cells which protrude away from the source. It has been proposed that this behavior represents an evolutionary foraging mechanism for vacating an environment where nutrient supplies have been exhausted.

The Swe1 protein is employed with similar utility when defects in bud formation are detected. When bud formation is impaired, either by mutation or by inhibitors of actin assembly, mitosis is delayed to allow for sufficient bud growth (Lew and Reed 1995). This mechanism also acts by blocking the degradation of Swe1 (McMillan *et al.* 1999). The septins are a group of conserved cytoskeletal proteins which form two rings flanking the future site of cell division in the bud neck (Kim *et al.* 1991; Longtine *et al.* 1996). Assembly of this septin ring is essential for proper bud growth (Barral *et al.* 2000). Failure to assemble the septin ring results in failure to activate the group of Nim1-related kinases that target Swe1 for degradation; thereby Swe1 is maintained in a stable, active state, occluding the onset of isotropic growth and mitosis (Barral *et al.* 1999; Shulewitz *et al.* 1999).

Recently an intriguingly analogous case has arisen in *Drosophila*: the *tribbles (trbl)* mutant exhibits failure in the proper temporal ordering of the mitotic domains during embryonic cell cycle 14 (Großhans and Wieschaus 2000; Mata *et al.* 2000; Seher and Leptin 2000). In all of the mitotic domains, save one, a pulse of $cdc25^{string}$ transcription immediately precedes mitosis (Edgar and O'Farrell 1989). The exception to this is domain 10 (the mesodermal precursor), where $cdc25^{string}$ expression initiates first in cycle 14. In *trbl* mutant embryos, this delay between $cdc25^{string}$ expression and mitosis in the mesodermal precursor is lost, and this domain enters mitosis before any of the others. This results in commencement of mitosis in the mesodermal precursor cells at essentially the same time as the morphogenetic movements of ventral furrow formation are normally occurring. Mitosis and gastrulation appear to be mutually exclusive processes, as this ectopic mitosis eliminates formation of the ventral furrow. The mechanism by which Trbl inhibits mitosis is not entirely clear, but has been shown to function at least in part through destabilizing the $Cdc25^{string}$ protein (Mata *et al.* 2000). *trbl* shows limited homology to the *nim1*-related family of kinases, although it appears to lack any functional kinase domain. It has been proposed that Trbl may also inhibit mitosis in a dominant-negative manner by serving as an indirect activator of Wee1: preventing its down-regulation or degradation by interfering with negative Wee1 regulators of the Nim1-like class (Großhans and Wieschaus 2000).

1.7 Meiosis

Unlike the mitotic cell cycle where DNA replication must precede nuclear division, the meiotic cycle negotiates two successive nuclear divisions following a single round of S phase. So the normal dependency relationship enforced in the mitotic cycle must be altered to allow for the 'equational' division of meiosis II. As outlined for the *Xenopus* meiotic cycle above, the meiocytes of many multicellular organisms remain in G2 or early prophase for an extended period of time during growth and development, and–particularly in higher eukaryotes–may remain in this state for months or even years. The oocyte is induced to mature (proceed through meiosis I and arrest in meiosis II) by a hormonal or environmental stimulus. Metaphase II arrest in mature oocytes is characterized by high MPF activity and stable cyclins.

The specific roles of Wee1 and Myt1 remain largely unknown. Meiosis and the early embryonic cell cycles in *Xenopus* are the clearest example of differential roles for these two related kinases. Myt1 is thought to maintain the prophase-like arrest in meiosis I, while Wee1 is absent at this time (Palmer *et al.* 1998). Progesterone activates the C-Mos/p42MAPK signaling cascade, which inactivates Myt1, contributing to the activation of MPF (Palmer *et al.* 1998; Peter *et al.* 2002). Additionally at this time, a Polo-like protein kinase (Plx1) activates Cdc25, commencing the autocatalytic amplification of Cdk1 activity (Qian *et al.* 2001). Ectopic expression of Wee1 during meiosis I results in ectopic entry into S phase following meiosis I rather than progression to the metaphase arrest of meiosis II (Nakajo *et al.* 2000). A low level of persistent Cdk1 activity following meiosis I is required to suppress the normally ensuing mitotic-like S phase (Iwabuchi *et al.* 2000).

The precise mechanism responsible for maintaining metaphase II arrest in the *Xenopus* oocyte has been a long-standing mystery. Cytoplasm from metaphase II arrested oocytes can cause other cells to arrest, and the entity conferring this activity was termed cytostatic factor, or CSF (Masui 1974). CSF activity maintains metaphase II arrest, and is abolished in the presence of divalent calcium ions, whose concentration spikes after fertilization (Meyerhof and Masui 1977; Meyerhof and Masui 1979; Shibuya and Masui 1982; Masui *et al.* 1984). CSF activity is dependent on activation of the C-Mos/p42MAPK pathway, signaling through p90Rsk (Sagata *et al.* 1989; Haccard *et al.* 1993; Bhatt and Ferrell 1999; Gross *et al.* 1999). MAPK pathway signaling inhibits the mitotic cyclin degrading activity of the APC/C/cyclosome (Reimann and Jackson 2002), and appears to do so by co-opting the spindle-assembly checkpoint protein Bub1 (Schwab *et al.* 2001b). In contrast to its effect on Myt1 in meiotic prophase I, the p42MAPK pathway *induces* Wee1 expression following fertilization and the completion of meiosis, and is responsible for the extended G2 phase of the first cell cycle (Murakami and Vande Woude 1998; Walter *et al.* 2000).

Unlike the case in *Xenopus*, *Drosophila* oocytes arrest in metaphase I instead of metaphase II. This arrest is dependent on the formation of DNA double strand breaks (DSBs), the necessary precursors to chiasmata, which interlink the homologous chromosomes and provide resistance to the tension of opposing spindles (McKim *et al.* 1993; Jang *et al.* 1995). Mutants that fail to initiate meiotic recombination fail to observe this arrest point, and proceed directly through meiosis.

During meiotic prophase, synapsis or pairing of homologous chromosomes occurs. This was originally thought to allow for the initiation of

DSBs and ensuing chiasmata formation, but this dependency relationship appears to have been inverted in some systems relative to others. Chiasmata represent the physical manifestation of crossing over and recombination. Contrary to the situation in budding yeast where synapsis is contingent on prior formation of DSBs (Alani *et al.* 1990; Engebrecht *et al.* 1990), meiotic chromosomes in female *Drosophila* can initiate synapsis in the absence of DSBs (McKim *et al.* 1998). Chiasmata may therefore serve dual functions: both in the form of purely mechanical facilitators of chromosome alignment and segregation, as well as from an evolutionary standpoint where crossing over provides for extensive 'shuffling' of genetic material, contributing to much of the diversity that is thought to have fueled the success of sexually reproducing organisms.

The enzymatically-induced DSBs required for crossing over must be detected and repaired just as DSBs induced in the mitotic cycle must be repaired before mitosis can proceed. Failure to detect and repair the DSBs induced in meiosis often gives rise to meiotic products with unbalanced genetic complements, rendering them inviable. This results in an apparent suppression of meiotic recombination. Several mutations that disrupt DNA damage signaling in mitosis also affect the meiotic DNA damage checkpoint (reviewed by Murakami and Nurse 2000). In both budding yeast and *Drosophila*, a Rad3-related protein is required for the meiotic DNA repair checkpoint (Hari *et al.* 1995; Lydall *et al.* 1996). Maternal mutants for weak alleles of *mei-41* are recombination defective. This is likely due to failure in detection and repair of DSBs, giving rise to gametes with deletions of genetic material (Hari *et al.* 1995). Likewise, in *S. cerevisiae*, mutations in recombination proteins required for repairing DSBs trigger the meiotic pachytene checkpoint, but this is abolished in combination with *mec1* (Lydall *et al.* 1996). In budding yeast, the meiotic DNA repair checkpoint acts through control of inhibitory phosphorylation of Cdc28 (as well as controlling Clb levels; Leu and Roeder 1999). *swe1* mutants are deficient for the meiotic checkpoint, as are cells with Cdc28(Y19F). Swe1 appears to be directly targeted and stabilized by the checkpoint, as its levels accumulate following checkpoint activation. This is in direct contrast to the mitotic checkpoint, and appears to resemble much more closely the mitotic checkpoint in *S. pombe.*

Studies in *Xenopus* indicate that a low level of Wee1 is present during meiotic prophase when DSBs are extant (Iwabuchi *et al.* 2000). In this case, inhibitory phosphorylation of Cdk1 by Myt1 may be necessary for enforcing this checkpoint. I examined the effect of a weak allele of *Dwee1* on meiotic recombination in *Drosophila* (see Appendix), which produces only a modest suppression of meiotic recombination. This could indicate that the mutant protein retains sufficient function to enforce the meiotic DNA damage checkpoint, or again that this function may be covered redundantly by *Dmyt1*.

1.8 Conclusion

In recent years, much knowledge has been gained about the role inhibitory phosphorylation of Cdk1 plays in the control of cell division, both in response to DNA checkpoints, as well as developmental signals. This progress has been aided immeasurably by sequence-based identification of structurally homologous genes across diverse species. The recent discovery of a checkpoint role for the *mus 304* gene, as well as the *trbl* gene in *Drosophila*, point to a potential wealth of specialized cell cycle regulatory mechanisms required in metazoan development. In both cases the extent of novelty is such that simple comparison to homologues of known cell cycle regulators would likely provide little or no insight to their function. For this reason it is still essential in the era of sequenced genomes that functional screens continue to be carried out in genetically tractable metazoan organisms.

In this work I describe the effects of loss of Wee1 kinase function in a metazoan organism, *Drosophila*. These observations provide us with a first glimpse into the role of Wee1-like kinases, both in development and the response to genomic insult in a multicellular organism. I also present a system to detect interacting mutations in genes which modulate the functions of Wee1-like kinases in development. I designed and oversaw the implementation of this system in a screen to search for dominant modifiers of *Dwee1* and *Dmyt1* overexpression phenotypes. Similar screens have proven fruitful in *Drosophila* for identifying novel regulatory factors.

In metazoan organisms the cell cycle machinery must not only be coordinated to intracellular events such as completion of S phase and DNA repair, but during the development of a multicellular structure, must be equipped to respond to signals from surrounding cells, both to initiate cell divisions, morphogenetic movements and differentiation on cue, as well as inducing cell death. We can anticipate that a multitude of novel regulatory mechanisms may be required as specialized adaptor modules to augment control of the basic cell cycle machinery in the process of development.

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2 *Drosophila wee1* Has an Essential Role in the Nuclear Divisions of Early Embryogenesis[*]

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2.1 Introduction

The nuclear division cycles of early *Drosophila* embryogenesis provide a formidable challenge to the cell cycle regulatory machinery. Initially, these cycles consist of extraordinarily rapid oscillations between S phase (2–3 min) and mitosis (5–6 min) that utilize maternally provided protein and RNA and occur within a syncytium. Interphase length then increases progressively during cycles 10–13 prior to the midblastula (MBT) or maternal-zygotic (MZT) transition in cycle 14 that precedes the onset of gastrulation (Foe and Alberts 1983). During normal embryogenesis, the developmentally regulated lengthening of interphase during cycles 10–13 requires maternally provided *mei-41* and *grp* encoded kinases (Sibon *et al.* 1997; Sibon *et al.* 1999). These genes encode homologs of the evolutionarily conserved checkpoint kinases Rad3/ATM and Chk1 (Hari *et al.* 1995; Fogarty *et al.* 1997). In fission yeast and humans, these kinases are components of a premitotic checkpoint that becomes activated in response to incompletely replicated or damaged DNA (Jimenez *et al.* 1992; Walworth *et al.* 1993; Beamish *et al.* 1996; Bentley *et al.* 1996; Walworth and Bernards 1996; Sanchez *et al.* 1997). Activation of the checkpoint is associated with inhibitory phosphorylation of Cdk1 (the central mitotic regulatory kinase) on a conserved tyrosine residue (Y15) by Wee1 kinases (O'Connell *et al.* 1997; Rhind *et al.* 1997; Rhind and Russell 1998). In response to DNA damage, Chk1 becomes activated (in a Rad3-dependent fashion) and phosphorylates Cdc25 on a residue that promotes Cdc25 interaction with members of the 14-3-3 family of proteins (Peng *et al.* 1997; Sanchez *et al.* 1997; Zeng *et al.* 1998; Chen *et al.* 1999; Lopez-Girona *et al.* 1999). This interaction prevents Cdc25 phosphatase from interacting with and activating Cdk1 by removal of Wee-catalyzed inhibitory phosphorylation. Consequently, either loss of Chk1 function or loss of Wee1 function can compromise the premitotic checkpoint.

The involvement of *mei-41* and *grp* in the slowing of the early embryonic cycles is proposed to reflect activation of a DNA replication checkpoint once maternally provided replication functions become limiting (Fogarty *et al.* 1997; Sibon *et al.* 1997; Sibon *et al.* 1999). The conservation of these checkpoint genes among eukaryotes implies that the biochemical mechanism by which they function might also be conserved. If so, it suggests that *Drosophila* embryos employ inhibitory phosphorylation of Cdk1 by a Wee1-like kinase to coordinate this developmentally regulated checkpoint. Inhibitory phosphorylation of maternal Cdk1 is not detected in *Drosophila* embryo extracts during cycles 10–13 when the *mei-41/grp* pathway is required, however (Edgar *et al.* 1994). This observation may indicate that *Drosophila* embryos employ the *mei-41/grp* pathway in a novel mechanism that is independent of Wee1-mediated inhibitory phosphorylation of Cdk1 to lengthen the syncytial cycles. We undertook a genetic analysis of *Dwee1*, a gene that encodes a *Drosophila* Cdk1 inhibitory kinase, to investigate these possibilities (Campbell *et al.* 1995).

2.2 Materials and Methods

2.2.1 Mutagenesis screen for *Dwee1* mutants

cn/cn males were mutagenized with either ethyl methanesulfonate (EMS; 25–50 mM) or diepoxybutane (DEB; 5 mM) according to standard protocols and then mated *en masse* to *Sco/CyO, cn* virgin females. These flies were transferred onto fresh media daily for 4–5 days, whereupon mutagenized males were removed. F1 progeny males carrying isolated mutagenized second chromosomes were collected and crossed individually to *Df(2L)Dwee1*^{W05}*/CyO, cn* virgin females (the origin and characterization of this deletion is described in the Results section). The F2 progeny were then scored for presence of the *cn/Df(2L)Dwee1*^{W05} class. Absence of this class indicated recovery of a zygotic lethal mutation (lethal alleles were designated *D*L or *E*L, depending on whether DEB or EMS was the relevant mutagen). In crosses where viable hemizygous F2 progeny were obtained, females of this class were mated to siblings to test for fertility (female-sterile alleles were designated *D*S or *E*S, depending on whether DEB or EMS was the relevant mutagen). Mutant stocks were established by mating of retained *cn/CyO, cn* siblings. Identified mutants were then further classified by complementation crosses with known mutants in the region and with *Df(2L)spd-J2*, a deletion whose published breakpoints are 27C1-28A (Neumann and Cohen 1996). Our genetic and molecular analysis of a stock carrying this deletion suggests that the distal breakpoint of this aberration is actually 27C4-5.

2.2.2 Transgene rescue experiments

A heat-shock-inducible construct was made by cloning the originally described *Dwee1* cDNA into the pCaSpeR-hs vector (Campbell *et al.* 1995). A genomic DNA construct that includes all of the *Dwee1* coding region plus upstream and downstream flanking DNA was constructed by cloning a ~10-kb *Hin*dIII fragment (sequence coordinates 14,273–24,263 in the DS01321 clone shown in Figure 2-1) into a pUAS vector (Brand and Perrimon 1993). For the inducible *Dwee1* rescue experiments, embryos were initially collected from mated *Dwee1*^{ES1} hemizygous females carrying a hs*Dwee1* transgene for 4 days without heat shocks. No viable embryos were observed. Flies were then heat-shocked in a 37° water bath once daily as indicated, collecting embryos at ~24-hr intervals (specific conditions are available on request). Fixed embryos (37% formaldehyde:heptane for 3 min) were then stained with Hoechst 33258 for analysis. Expression of the transgene in *Dwee1*^{ES1}*/hsDwee1, Df(2L)Dwee1*^{W05} adult females was confirmed by heat-shocking for 30 min (37°), with a 90-min recovery period. These flies were then homogenized in loading buffer (2.0% SDS, 60 mM Tris (pH 6.8), 0.01% bromophenol blue, 10% glycerol, and 0.1 M DTT) and the cleared extract was diluted in loading buffer prior to SDS-PAGE (8% acrylamide). Proteins were then transferred onto Hybond-P membrane and the membrane was probed with a rabbit anti-Dwee1 primary antibody (anti-DKD at 1:1600). The membrane was then reprobed with mouse anti-ß-tubulin (1:500; Amersham, Buckinghamshire, UK). Secondary antibody hybridization signal was detected using the ECL+ chemiluminescence system (Amersham).

2.2.3 Responses of *Dwee1* mutants to hydroxyurea

A genetic cross between *Dwee1*^{ES1}*/CyO* and *Df(2L)Dwee1*^{W05}*/CyO* flies was done, following which 24-hr embryo collections were made. After a further 24 hr of development, 1 ml of aqueous hydroxyurea concentrate was added to the medium (values in Figure 2-5 indicate final concentration). Distilled water (1 ml) was substituted for controls. Adult flies were scored daily once they began to eclose, to completion.

2.2.4 Genomic sequencing and DNA analysis

Genomic DNA was extracted by standard techniques (Campbell *et al.* 1995) from hemizygous adult flies and used as a template for direct amplification using *Pfu* polymerase. The amplified product was then cycle-sequenced on both strands using the Thermosequenase system (Amersham). Mutations were confirmed by independent sequencing reactions. The GenBank accession number for *Dwee1* is U17223. Genomic DNA analysis to localize insertions and deletion breakpoints was also done as described in Campbell *et al.* 1995, using digoxygenin-labeled probes according to the manufacturer's recommendations (Boehringer Mannheim, Indianapolis).

2.3 Results

2.3.1 A screen for lethal and sterile mutations in genes uncovered by *Df(2L)Dwee1*^{WO5}

Previous studies of the *Dwee1* locus utilized a large deletion, called *Df(2L)Dwee1;27A-28B*, that uncovers *Dwee1* as well as a number of other genes (Campbell *et al.* 1995). These studies established that loss of zygotic *Dwee1* function does not produce a detectable mitotic phenotype in embryos. To generate a smaller deletion for further genetic studies of the locus, transposase-mediated imprecise excision of a nearby *P{w+}* transposon insertion, associated with *l(2)k10413*, was used to generate w- derivative chromosomes (see Figure 2-1). Both *l(2)k10413* and a nearby P insertion, *l(2)02647*, have been cytologically mapped to position 27C4-5 by the Berkeley *Drosophila* Genome Project (BDGP) and have been described as mutant alleles of the *hrp48* gene (also called *Hrb27C*) that encodes a ribonuclear splicing factor (Matunis *et al.* 1992; Hammond *et al.* 1997). Flies that are transheterozygous for these two alleles (*l(2)k10413/l(2)02647*) are viable but usually have small nicks in the wing margin. The w- derivatives generated by mobilization of the *P(w+)* insertion associated with *l(2)k10413* were thus initially screened by failure to complement *l(2)02647* for viability as a means of identifying potential excision events that extended toward the *Dwee1* locus. Stocks carrying noncomplementing alleles were then screened by genomic DNA analysis for molecular aberrations that would indicate the recovery of deletions uncovering the *Dwee1* coding region. A single deletion, *Df(2L)Dwee1*^{WO5}, was identified by this approach. This deletion uncovers the *Dwee1* locus and additional genes shown in Figure 2-1 that were identified by sequence comparisons of the completed genomic sequence of the region (P1 clone: DS01321, Berkeley *Drosophila* Genome Project) with the expressed sequence tag (EST) database. *Df(2L) Dwee1*^{WO5} is lethal in transheterozygous combinations with all previously characterized alleles of *hrp48* and with the single *P*-insertion allele of another gene of unknown function designated *l(2)k00213* (mapped to position 27C2-3 by BDGP). It also fails to complement *Df(2L)spd-J2* (Neumann and Cohen 1996). Homozygous *Df(2L)Dwee1*^{WO5} mutants derived from heterozygous parents die late in embryogenesis with no obvious mitotic abnormalities, consistent with previous characterization done with the larger deletion, *Df(2L)Dwee1;27A-28B*. A recombinant chromosome carrying *Df(2L)Dwee1*^{WO5} and a proximal flipase recombinase target (FRT) sequence was generated to investigate maternal requirements for *Dwee1* function (Chou and Perrimon 1996). No eggs were generated from *FLP*-expressing, *ovo*^{D}FRT/*Df(2L)Dwee1*^{WO5}FRT transheterozygous females for analysis, however, from which we could only conclude that one of the genes uncovered by the deletion must be essential for oogenesis (data not shown).

To further investigate the function of *Dwee1*, a chemical mutagenesis screen was devised to identify point mutations in genes within the region delimited by *Df(2L)Dwee1*^{WO5}. A standard F2 screening protocol was followed as described in the Materials and Methods section. Hemizygous F2 progeny were tested for zygotic lethality and those that were viable were then tested for female sterility. By this approach we identified four lethal and three female-sterile mutants from progeny representing ~4500 individual candidate chromosomes.

2.3.2 Characterization of lethal alleles recovered in the screen

Complementation tests established that the first lethal mutation recovered in our screen (*l(2)EL1*) is an allele of the gene thought to be associated with the *P*-transposon insertion designated *l(2)k00213* (Torok *et al.* 1993). We have not yet determined which gene within or overlapping *Df(2L)Dwee*^{WO5} is affected by these mutations, but there are clearly several potential candidates (Figure 2-1).

Df(2L)spd-J2, previously characterized as uncovering cytological interval 27C1-28A, was crossed to all of the mutations recovered in our screen as well as to known P-element mutations in the region (Neumann and Cohen 1996). This deletion failed to complement lethal P-insertion alleles of the *hrp48* locus [*l(2)02647* and *l(2)k10413*] as well as our new lethal mutation called *l(2)EL2*, but does complement all other mutations described in this study except *Df(2L)Dwee1*^{WO5}. These observations prompted us to determine the distal breakpoint of this deletion using molecular techniques. We found it within an interval just distal to *hrp48*, as indicated in Figure 2-1.

The complementation patterns of two other lethal alleles recovered in our screen, *l(2)EL3* and *l(2)DL1*, are complex (see Table 2-1). Both complement the two *P*-insertion alleles of *hrp48* as well as *Df(2L)spd-J2*, suggesting that these mutations have not disrupted the *hrp48* locus. However, our new *hrp48* allele *l(2)EL2* failed to complement *l(2)EL3*. Furthermore, while *l(2)DL1* complemented all the *hrp48* alleles we tested, it failed to complement *l(2)EL3*. We are not able to resolve the genetic basis for these peculiar genetic interactions at this time. One possibility is that our data reflect interallelic complementation among different alleles of a complex genetic locus. Various cDNA clones characterized by the BDGP from the *hrp48* locus fall into three distinct classes of splice products, possibly accounting for the observed genetic complexity (see Figure 2-1).

2.3.3Characterization of *Dwee1* female-sterile mutations recovered in the screen

Complementation tests showed that all three female-sterile mutations recovered in our screen (*Dwee1*^{ES1}, *Dwee1*^{ES2}, and *Dwee1*^{DS1}) are alleles of the same gene, and data described later in this section establish that this gene corresponds to *Dwee1*. We undertook a detailed phenotypic analysis of one of the mutant alleles, *Dwee1*^{ES1}. Hemizygous *Dwee1*^{ES1} mutant females are viable but completely sterile and show no paternal rescue effect (hemizygous males are fertile, however). Hemizygous females lay abundant eggs of normal appearance that proceed through the early syncytial nuclear cycles without incident. During cycles 11 and 12, however, nuclei in mutant-derived embryos fail to separate at the end of mitosis and remain fused (Figure 2-2). This phenotype and the subsequent clumping and fragmentation of nuclei that we observe (Figure 2-3C) is identical to what is seen in embryos collected from *grp* or *mei-41* mutant females (Fogarty *et al.* 1994; Sibon *et al.* 1999). This observation suggests a possible role for *Dwee1* in the same developmental checkpoint as *mei-41* and *grp*.

We undertook two different approaches to demonstrate that the complementation group represented by the three female-sterile mutations does in fact correspond to *Dwee1*. First, we sequenced genomic DNA isolated from adults hemizygous for each of the alleles (*Dwee1*^{ES1}, *Dwee1*^{ES2}, and *Dwee1*^{DS1}), covering the entire transcribed region and ~150 bp of flanking DNA at each end. For comparison, we sequenced genomic DNA from the stock used to generate the mutants. With respect to their maternal phenotype, *Dwee1*^{ES1} and *Dwee1*^{ES2} behave as classical amorphic alleles (Muller 1932). Genomic DNA isolated from each of these mutants contains a DNA lesion within the kinase domain of *Dwee1* that is expected to either abolish or severely disrupt the function of the gene (Figure 2-4). *Dwee1*^{ES1} contains an 8-bp deletion causing a frameshift followed by a stop codon, truncating the protein in kinase domain IV. *Dwee1*^{ES2} contains a missense mutation that changes a glutamate residue that is conserved among Wee1-like kinases to a lysine at position 308 in the protein (E308K). *Dwee1*^{DS1} behaves as a classical hypomorphic allele in that the phenotype of embryos derived from homozygous females is much less severe (many cellularize and some even develop to adulthood) than that of embryos derived from hemizygous females (which rarely cellularize and never hatch). Sequence analysis of this allele showed that it contains a missense mutation changing a conserved phenylalanine residue to isoleucine at amino acid residue 250 within the ATP-binding site of the protein (F250I). Presumably this lesion is still compatible with low-level function of the protein. The *Dwee1*^{ES1} allele shows an antimorphic interaction with the *Dwee1*^{DS1} allele in that the phenotype of embryos derived from *Dwee1*^{DS1}/*Dwee1*^{ES1} transheterozygous mothers is more severe (embryos never cellularize) than seen in *Dwee1*^{DS1}/*Df(2L)Dwee1*^{WO5} hemizygotes. Conceivably, this reflects titration of positive regulatory factors by the truncated *Dwee1*^{ES1} protein, thus lowering the effective levels of *Dwee1*^{DS1} function.

We were also able to partially rescue the phenotype of mutant embryos with a heat-inducible *Dwee1* cDNA transgene. Maternal *Dwee1*^{ES1} hemizygous flies carrying this transgene were briefly heat-shocked to induce expression as confirmed by immunoblot analysis (Figure 2-3A). Rescue was scored as development at least to the cellularization stage (cycle 14), which mutant-derived embryos otherwise never reach. By this measure, ~50% of the embryos could be rescued by maternal expression of the transgene. Cessation of heatshocks produced a decline in numbers of rescued embryos (Figure 2-3B). We observed wide phenotypic variation in the extent of phenotypic rescue, presumably reflecting variations in the amount and timing of Dwee1 protein and mRNA deposited into individual eggs. These ranged from mosaic embryos containing both cellularized and syncytial sectors to apparently normal late embryos and first instar larvae that were nonetheless unable to complete development (Figure 2-3C). In contrast, heat-shock treatment of *Dwee1*^{ES1} hemizygous females lacking the transgene produced no cellularized embryos, eliminating the possibility that our mutants were being rescued by the experimental protocol alone. Furthermore, a single transgene copy of a genomic DNA construct that contains *Dwee1* coding sequences plus flanking DNA (and includes the adjacent *dhp1-like* gene) can completely rescue the maternal lethal phenotype. These two lines of evidence demonstrate that molecular lesions consistent with loss of function in *Dwee1* are found in the female-sterile mutants and also show that *Dwee1* expression is both necessary and sufficient to rescue the maternal lethal phenotype. We conclude from this evidence that we have identified mutant alleles of *Dwee1*. The striking similarity between the phenotype of *Dwee1* mutant-derived embryos and embryos derived from *grp* or *mei-41* mutants provides a strong argument that maternally provided *Dwee1* plays an essential role in the same developmental process as *grp* and *mei-41*.

Additional evidence in favor of this hypothesis is afforded by providing extra maternal copies of the genomic *Dwee1* transgene in a *mei-41*^{D3} mutant background. Females homozygous for the *mei-41*^{D3} allele produce cellularized embryos at a very low frequency (2%, N = 106). The frequency of cellularized embryos is dramatically increased by adding an extra maternal copy of a *Dwee1* genomic transgene (20%, N = 109). The *mei-41*^{D3} mutant embryos are further rescued by addition of two *Dwee1* transgenes (50%, N = 72), to the extent that some *mei-41*^{D3}-derived embryos were able to develop to adulthood. In contrast, parallel experiments in a *grp*^{1} background did not produce any rescue of the mutant phenotype with either one or two extra copies of *Dwee1*. The simplest interpretation we can offer for why the results differ between *grp* and *mei-41* mutants in these experiments is that the *mei-41*^{D3} is not a complete loss-of-function allele, and consequently *mei-41*^{D3} mutants are more sensitive to increased dosage of *Dwee1* than *grp1* mutants. Alternatively, *grp* may respond to two different signaling pathways whereas *mei-41* may respond to only one of the two. Dwee1 overproduction could be sufficient to rescue the common function but not the *grp*-specific one according to this model. Another test for functional interactions among these genes was to assess the effect of lowering the maternal dosage of *mei-41+* or *grp+* in a homozygous *Dwee1*^{DS1} maternal background. The incompletely penetrant syncytial arrest phenotype of homozygous *Dwee1*^{DS1}-derived embryos (54% cellularized, N = 107) was enhanced by subtracting a maternal copy of *mei-41+* (39%, N = 141). Removal of one maternal copy of *grp+* produced an even greater enhancement of the mutant phenotype of *Dwee1*^{DS1}-derived embryos (29% cellularized, N = 127).

We wanted to assess whether *Dwee1* hemizygous flies derived from heterozygous parents were capable of mounting an effective response to delays in DNA replication, since the slowing of the late syncytial cycles has been proposed to reflect activation of a DNA replication checkpoint (Sibon *et al.* 1997; Sibon *et al.* 1999). For this experiment, we assessed the sensitivity of *Dwee1*^{ES1} hemizygous larvae to treatment with hydroxyurea (HU), a drug that inhibits DNA replication. In fission yeast, the "checkpoint rad" group of mutants as well as *wee1* mutants are all extremely sensitive to HU. In *Drosophila*, *mei-41* and *grp* mutant larvae also exhibit this response (Sibon *et al.* 1999). Genetic crosses between balanced heterozygous stocks carrying either the *Dwee1*^{ES1} mutant chromosome or the *Df(2L)Dwee1*^{W05} chromosome generate both heterozygous and hemizygous viable adult progeny. Exposure to 1 or 2 mM HU eliminated the hemizygous *Dwee1*^{ES1} class of progeny, indicating that *Dwee1* mutant larvae are indeed highly sensitive to HU, presumably reflecting a requirement for *Dwee1* activity in a fully functional DNA replication checkpoint (Figure 2-5).

2.4 Discussion

These studies establish that *Dwee1* has an essential maternal function during the nuclear division cycles of embryogenesis and also implicate zygotic *Dwee1* function in a cell cycle checkpoint that responds to inhibition of DNA replication. The demonstration that *Dwee1* has a role during the early syncytial nuclear cycles calls into question a previous assumption that inhibitory phosphorylation does not control these cycles. Analyses of the state of phosphorylation during the early cycles had failed to detect inhibitory phosphorylation of Cdk1 prior to cycle 13 (Edgar *et al.* 1994). Furthermore, because reduction in the gene dose of *cyclin A* and *cyclin B* slowed the late nuclear cycles, it was suggested that progress of these cycles is regulated by accumulation of cyclins to a threshold level. The finding that *Dwee1* is required for completing the nuclear division cycles suggests that inhibitory phosphorylation does play a role in their regulation after all. The failure to detect inhibitory phosphorylation during these cycles can be explained if only a small pool of Cdk1 is subject to this modification. Wee1-type kinases are predominantly nuclear in *Drosophila* and other organisms and nuclear Wee1 activity is sufficient to block entry into mitosis even in the presence of high cytoplasmic Cdk1 activity (Heald *et al.* 1993; Campbell *et al.* 1995). Hence, we suggest that inhibitory phosphorylation of a small nuclear pool of Cdk1 contributes importantly to the control of the syncytial cycles. The proposal that inhibitory phosphorylation regulates syncytial cycles was an implicit component of a recently proposed model for the mechanism by which *mei-41* and *grp* regulate the progressive lengthening of these cycles (Sibon *et al.* 1997; Sibon *et al.* 1999). In response to incompletely replicated DNA, the recognized activities of these conserved checkpoint kinases arrest the cell cycle by preventing the removal of inhibitory phosphates from Cdk1. While this model appears to be at odds with the lack of detectable inhibitory phosphorylation of Cdk1 during the syncytial cycles (Edgar *et al.* 1994), our findings that *Dwee1* is required for the early nuclear division cycles supports this proposal. Indeed, the apparent parallels in the phenotypes of *mei-41*, *grp*, and *Dwee1* maternal mutants suggest that these genes operate by a similar mechanism. Because our results implicate this pathway without defining precisely how it is induced, it remains possible that the same pathway could be used in a unique regulatory circuit, as was recently suggested (Su *et al.* 1998). In either case, the lesson seems to be that the remarkable conservation of the eukaryotic cell cycle regulatory machinery is coupled with an equally remarkable flexibility in how that machinery can be deployed, depending on the particular developmental constraints of each organism. In early *Drosophila* embryos, a regulatory pathway that usually serves a surveillance function plays an essential cell cycle role.

It was unexpected that zygotic *Dwee1* function would be dispensable under normal growth conditions, since Cdk1 inhibitory phosphorylation appears to play an important role in cell cycle regulation at many stages of development in *Drosophila*. Following the last syncytial division during interphase of cycle 14, Cdk1 becomes quantitatively inhibited by phosphorylation (Edgar *et al.* 1994). This dramatic regulatory transition could result from delocalization of Dwee1, activation of a cytoplasmically localized Cdk1 inhibitory kinase, inhibition of cytoplasmic Cdc25, or more active exchange of Cdk1 between the nucleus and cytoplasm during cycle 14. We are currently investigating these possibilities. It has been demonstrated that entry into mitosis 14 depends on zygotic expression of Cdc25^{stg} phosphatase and removal of inhibitory phosphate from Cdk1 (Edgar and O'Farrell 1989; Edgar and O'Farrell 1990). Furthermore, Cdc25^{stg} activity is also required during the following postblastoderm mitoses of embryogenesis and during imaginal disc development (Edgar and O'Farrell 1989; Edgar and O'Farrell 1990; Milan *et al.* 1996; Johnston and Edgar 1998). Cdc25^{twe} activity is also required during meiosis (Alphey *et al.* 1992; Courtot *et al.* 1992). These requirements for Cdc25^{stg} imply that inhibitory phosphorylation is normally significant at all of these stages of development. In fission yeast, loss of Wee1 kinase can suppress requirements for the Cdc25 phosphatase. In *Drosophila*, however, loss of zygotic *Dwee1* function does not bypass the requirement for Cdc25^{stg} activity (Campbell *et al.* 1995). The continued requirement for Cdc25stg activity might be due to maternal perdurance of *Dwee1* function. Alternatively, there might be other Wee1 kinases that can function either redundantly with *Dwee1* or independently. We have recently cloned the gene encoding a *Drosophila* homolog of Myt1, a Wee1-related kinase, which may contribute to some of these activities (Mueller *et al.* 1995; Cornwell *et al.* 2002).

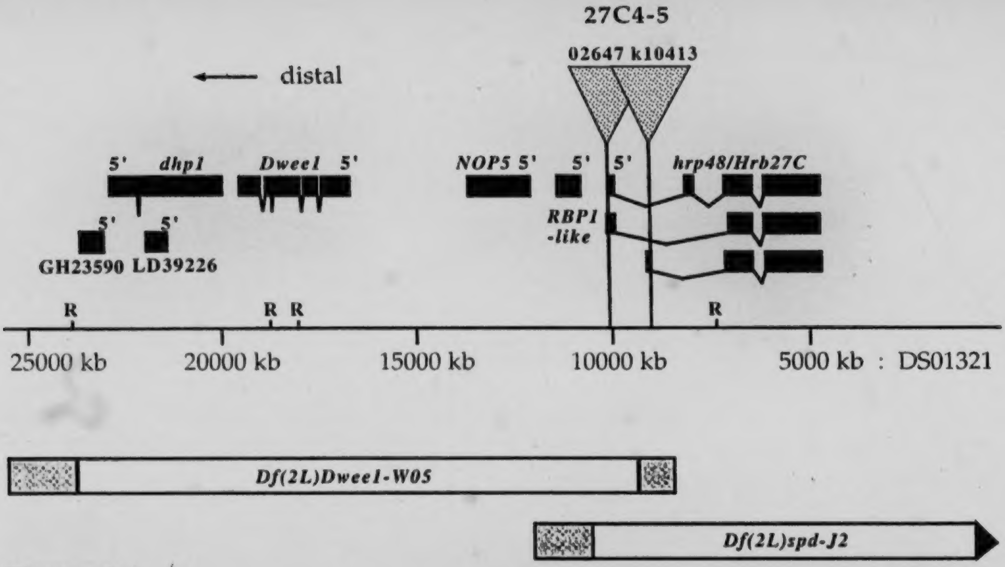

Figure 2-1
Genome organization of cytological region 27C4-5 with respect to transcribed genes and the extent and position of deletions and *P*-transposon insertions described in the text. The open horizontal boxes indicate the extent of the two deletions that impinge on this region, with shaded boxes representing regions within which the deletion endpoints were molecularly mapped by genomic hybridization analysis. The numbered coordinates represent genomic DNA sequence positions within the DS01321 clone. The positions of sites for restriction enzyme *Eco*RI are indicated (R) on the horizontal line representing the genomic sequence as a further orientation guide. Triangles connected to the sequence by vertical lines represent the insertion sites of two P-transposon insertion alleles of the *hrp48* locus that are described in further detail in the text. BDGP determined the indicated cytological and molecular positions of these P-transposon insertions. Solid horizontal lines represent the exon/intron organization and orientation of transcripts identified in the region that correspond to previously sequenced ESTs in the BDGP database. ESTs with open reading frames whose translation products show high sequence similarity to proteins identified in other organisms are named with respect to those homologs. GH23590 and LD39226 represent ESTs that do not show significant homology to other known genes and may correspond to exons of the same gene. The transcript designated *dhp1* is homologous to a gene implicated in recombination and RNA metabolism called *dhp1/RAT1/Dhm1* in *Schizosaccharomyces pombe, Saccharomyces cerevisiae*, and mice, respectively (Kenna *et al.* 1993; Sugano *et al.* 1994; Shobuike *et al.* 1995). The transcript designated *NOP5* is homologous to a gene of the same name implicated in nucleolar assembly in *S. cerevisiae* (Wu *et al.* 1998). The transcript designated *RBP1-like* is similar to a member of the SR protein family of splicing accessory factors required for sex-specific splicing of *doublesex* (Heinrichs and Baker 1995; Lynch and Maniatis 1996). The genes designated *Dwee1* and *hrp48* are described in further detail in the text.

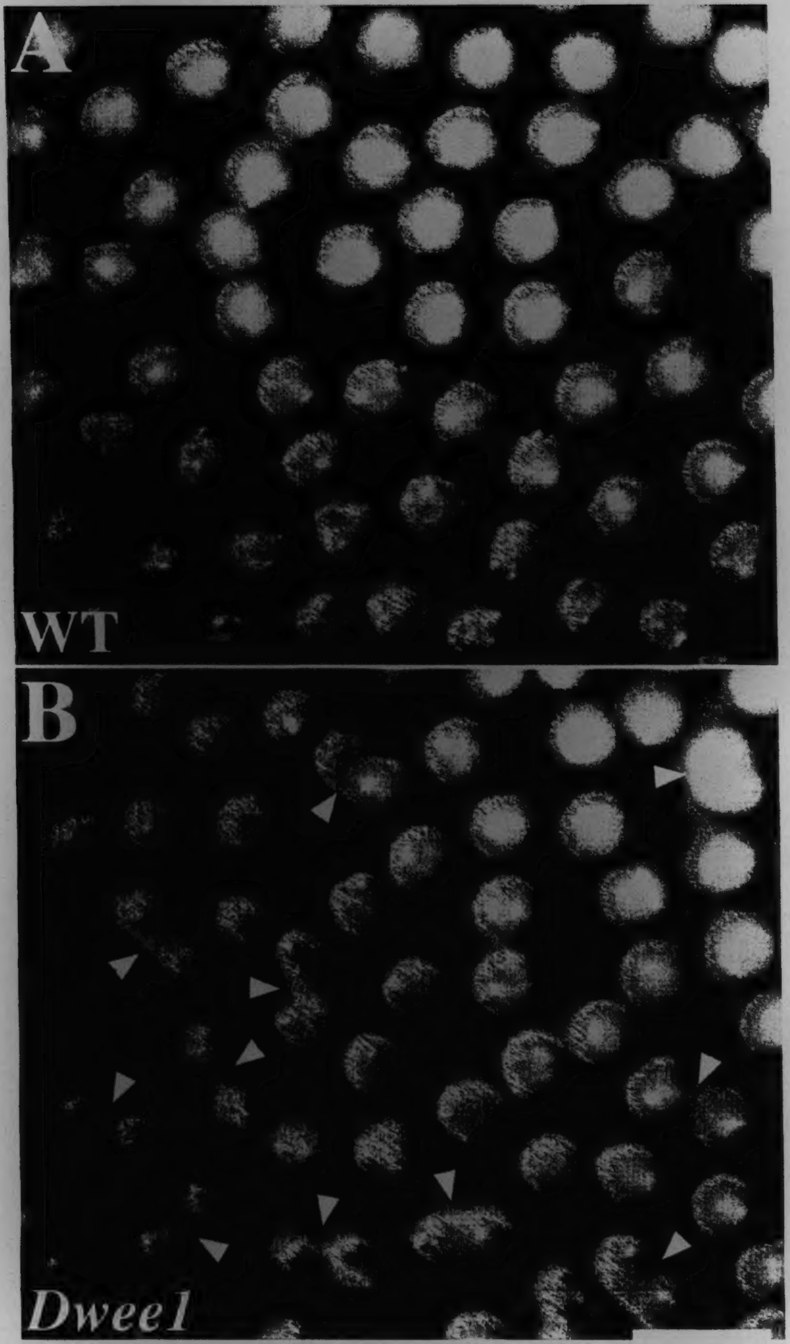

Figure 2-2
Comparison of Hoechst 33258-stained nuclei from equivalently staged wild-type and *Dwee1* mutant embryos. (A) Nuclei from a wild-type embryo in interphase of cycle 13. (B) Nuclei from a *Dwee1*-derived embryo in interphase of cycle 13. The arrowheads indicate nuclei that have failed to divide in mitosis 12. Bar, 12 µm.

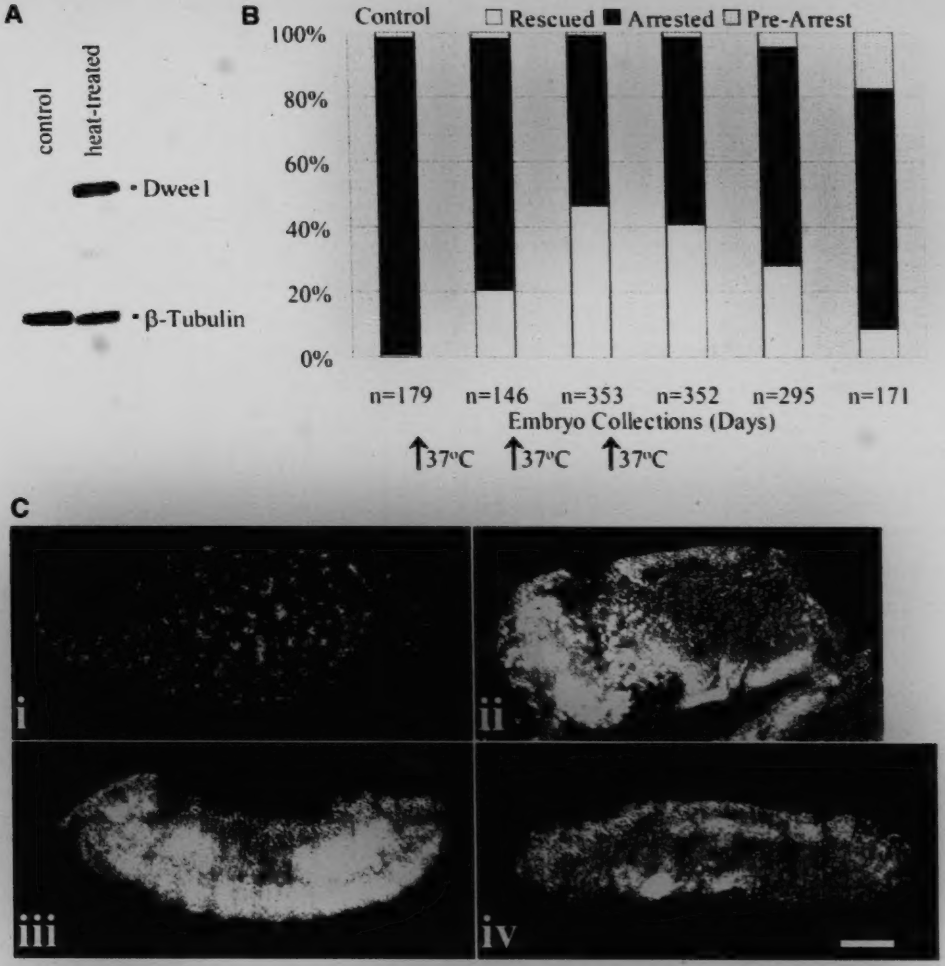

Figure 2-3
Expression of a *hsDwee1* transgene can rescue the maternal lethal phenotype of *Dwee1* mutant-derived embryos. (A) Immunoblot showing induction of Dwee1 protein by heat shock of *Dwee1*^{ES1}/*hsDwee1*, *Df(2L)Dwee1*^{W05} adult female flies. (B) Classes of embryos produced by *Dwee1*^{ES1}/*hsDwee1*, *Df(2L)Dwee1*^{W05} mothers. Numbers of embryos counted for each collection are given at the bottom of the graph. The bar marked "control" represents embryos collected prior to induction of the transgene. Embryos that were completely syncytial and displayed the typical nuclear defects described for *Dwee1* mutants were scored as "arrested." Embryos were scored as rescued if they were able to complete syncytial development and cellularize (at least partially). Embryos that were developmentally too early to score as being either arrested or rescued were scored as "pre-arrest." (C) Variation in embryonic phenotypes observed in the heat-shock rescue experiment. (i) Syncytial arrest phenotype typical of *Dwee1*-derived embryos. (ii) Mosaic embryo with both cellularized (right) and arrested (left) domains. (iii and iv) Apparently normal late embryo and early larva, respectively. Bar, 62 µm.

GV**GEFG**VV**F**Q**CV**N**RLDG**CI**YAIK**K**SKK**PV**AGS**SF**E**KRA**L**N**EV**W**AH**

↓

I (DS1)   DGGRSYADTE<STOP> (ES1)

↑

AVLGK**H**DN**VVRY**Y**SAWAEDDHM**L**IQNE**F**C**D**GGSL**HAR**I**QDHCLG**E**

↓

K (ES2)

A**ELKI**V**L**MH**V**IE**GLRYIH**SND**LVHMDLKPENIF**S**T**MNPNAHKLVE

VQPQQTKD**DD**GMDSVYEELRHSENL**V**TY**KIGDLGHVT**S**V**KEPY**VE**

EGDC**RYL**PK**EIL**H**E**D**Y**SN**L**F**KADIFSLG**I**T**LF**EAAG**GG**PLP**K**NG**P

E**WH**N**LR**D**G**KV**P**IL**P**S**L**

Figure 2-4
Location of amino acid changes in the Dwee1 protein, inferred from molecular lesions identified in genomic DNA of the *Dwee1* mutants identified in this study. The sequence shown represents amino acid residues 246 to 486 of the *Dwee1* coding region and the letters denote amino acids in the standard single-letter designation. Letters in bold signify residues that are conserved in other Wee1 kinases. The arrows indicate the position of lesions identified in the *Dwee1*^{ES1} (ES1) and *Dwee1*^{ES2} (ES2) EMS-induced and *Dwee1*^{DS1} (DS1) DEB-induced mutant alleles, respectively, and point to the altered residues that the mutant alleles are predicted to encode.

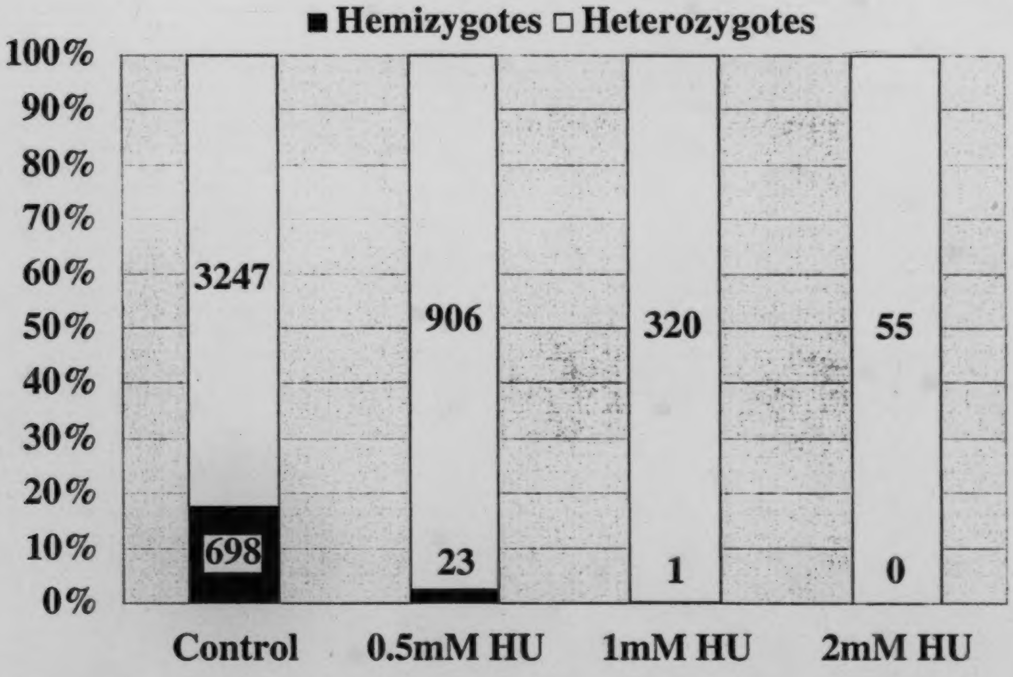

Figure 2-5
Dwee1 mutants are defective for a zygotic DNA replication checkpoint. The graph shows populations of progeny from *Dwee1*^{ES1}/*CyO* flies crossed with *Df(2L)Dwee1*^{W05}/*CyO* flies that were grown in vials containing the indicated concentration of hydroxyurea. The visible adult marker *Cy* carried on the second chromosome balancer in the heterozygotes was used to distinguish the genotypes of hemizygous and heterozygous flies.

Summary of complementation at the *hrp48/Hrb27C* locus

Allele	*l(2)02647*	*l(2)k10413*	*l(2)EL2*	*l(2)EL3*	*l(2)DL1*
l(2)02647	Lethal				
l(2)k10413	Semilethal	Lethal			
l(2)EL2	Lethal	Lethal	ND[a]		
l(2)EL3	Viable	Viable	Lethal	ND	
l(2)DL1	Viable	Viable	Viable	Lethal	ND
Df(2L)spd-J2	Lethal	Lethal	Lethal	Viable	Viable
Df(2L)Dwee1^{W05}	Lethal	Lethal	Lethal	Lethal	Lethal

[a] Allele combinations marked "ND" are lethal but are treated as uninformative since it is likely these chromosomes harbor second-site lethal mutations.

Table 2-1

2.5 Bibliography

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3 Ectopic Expression of the *Drosophila* Cdk1 Inhibitory Kinases, Wee1 and Myt1, Interferes With the Second Mitotic Wave and Disrupts Pattern Formation During Eye Development*

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3.1 Introduction

The control of mitosis by inhibitory phosphorylation of cyclin-dependent kinase (Cdk)1 has been characterized extensively in unicellular eukaryotes. In *Schizosaccharomyces pombe*, signaling pathways responsive to cell size, DNA damage, and DNA replication target the phosphorylation of Cdk1 residue tyrosine 15 (Y15), thereby functioning to maintain genome integrity (Rhind *et al.* 1997; Rhind and Russell 1998). Inhibitory phosphorylation of Cdk1 is catalyzed by both Wee1 and Mik1 kinases in *S. pombe* (Russell and Nurse 1987b; Featherstone and Russell 1991; Lundgren *et al.* 1991; Lee *et al.* 1994) and is reversed by Cdc25 and Pyp3 phosphatases (Russell and Nurse 1986; Gould *et al.* 1990; Millar *et al.* 1991; Millar *et al.* 1992). In contrast, inhibitory phosphorylation of a Cdk1 homologue (Cdc28) is not required for maintenance of genome integrity in *Saccharomyces cerevisiae* (Amon *et al.* 1992; Sorger and Murray 1992). Instead, a *SWE1*-mediated checkpoint delays mitosis by inhibiting Cdc28 in response to defective assembly of the actin cytoskeleton and promotes filamentous growth when nutrients are limiting (Lew and Reed 1995; Sia *et al.* 1996; McMillan *et al.* 1998; Sia *et al.* 1998; Barral *et al.* 1999; Edgington *et al.* 1999).

During *Drosophila* embryogenesis, inhibitory phosphorylation of Cdk1 is required for maintaining G2 phase during the embryonic cell divisions. Expression of *cdc25*^{string} overcomes this inhibition, inducing mitosis in spatially and temporally patterned mitotic domains (Edgar and O'Farrell 1990). The intricate pattern of *cdc25*^{string} transcription is governed by *cis* elements in a large regulatory region that integrates a diverse array of patterning gene inputs to direct the appropriate spatiotemporal pattern of *cdc25*^{string} expression during embryonic and imaginal development (Edgar *et al.* 1994; Johnston and Edgar 1998; Lehman *et al.* 1999). Heat shock expression of a constitutively active, nonphosphorylatable Cdk1 variant (Cdk1AF) is lethal to *Drosophila* embryos, indicating that inhibitory phosphorylation of Cdk1 is essential for regulating mitosis during development; however, regulation of a similar S phase kinase (Cdk2) on a conserved tyrosine residue is not (Lane *et al.* 2000).

In metazoans, two adjacent inhibitory phosphorylation sites on Cdk1 (T14 and Y15) are substrates for two distinct Wee1-like kinases that differ in their subcellular localization. Nuclear Wee1 kinases phosphorylate Y15 exclusively, whereas Myt1, a membrane-localized Wee1-like kinase, can phosphorylate either site (Kornbluth *et al.* 1994; Mueller *et al.* 1995; Booher *et al.* 1997; Liu *et al.* 1997). The physiological significance of these differences between the Wee1 and Myt1 kinases is presently unknown. We are addressing this question by characterizing the functions of Wee1 and Myt1 kinases during *Drosophila* development. *Drosophila* encodes a single *wee1* homologue (*Dwee1*), originally identified by its ability to complement a lethal mitotic catastrophe phenotype in *S. pombe* cells that were mutant for both *wee1* and *mik1* (Campbell *et al.* 1995). Null alleles of *Dwee1* are maternal effect lethal and *Dwee1*-derived embryos undergo catastrophic nuclear defects during the late syncytial divisions that include failure to complete nuclear division (Price *et al.* 2000) and failure to lengthen interphase, as normally occur when a developing embryo approaches cycle 14 (This work—see Appendix). The phenotype of *Dwee1*-derived mutant embryos is similar to phenotypes of maternal mutants for *mei-41* or *grapes* (*grp*), the *Drosophila* homologues of the checkpoint kinases *rad3/ATR* and *chk1*, respectively (Fogarty *et al.* 1994; Fogarty *et al.* 1997; Sibon *et al.* 1997; Sibon *et al.* 1999). These phenotypic similarities suggest that the three genes act in a common checkpoint pathway during early embryonic development, an idea supported by genetic interactions between mutant alleles of these genes (Price *et al.* 2000).

Given the critical importance of inhibitory phosphorylation during embryogenesis, it was puzzling that the zygotic function of *Dwee1* is not essential and that *Dwee1* mutants develop normally under ordinary circumstances. *Dwee1* mutant larvae do die when they are fed hydroxyurea at concentrations that wild-type larvae can tolerate, however, apparently due to a defective DNA replication checkpoint (Price *et al.* 2000). The viability of zygotic *Dwee1* mutants could be due to the presence of a redundant Cdk1 inhibitory kinase such as Myt1. Although cellular localization and substrate specificity differences suggest that Wee1 and Myt1 homologues serve distinct roles in cell cycle regulation, the two metazoan Wee1-like kinases may also share some redundant functions, as *wee1* and *mik1* do in *S. pombe* (Lundgren *et al.* 1991). To investigate this possibility we cloned the single *Myt1*-like gene from *Drosophila*, *Dmyt1*, and are undertaking a genetic analysis of its function during development.

In this report we describe phenotypic defects caused by overexpressing either *Dwee1* or *Dmyt1* in developing tissues. Overexpression in the eye imaginal disc causes visible defects in the adult eye. The eye phenotype can be modified by mutations in known cell cycle regulators, suggesting that this system might be capable of detecting previously uncharacterized mitotic regulators that have evolved to coordinate cell proliferation with specific developmental events. We have tested this idea by screening for dominant genetic modifiers, using a collection of deletions comprising 70–80% of the *Drosophila* euchromatic genome. These tests have identified several loci that potentially encode novel regulators of either Wee1 or Myt1.

3.2 Materials and Methods

3.2.1 Cloning of the *Drosophila Myt1* gene

A small fragment of *Dmyt1* was amplified by PCR using degenerate primers designed against conserved regions of *Xenopus* and human *Myt1* (CKLGDFG and AADVFSL). After sequencing to confirm that we had in fact isolated a genomic sequence that was similar to the *Myt1* homologues, the PCR fragment was labeled and used to screen the pNB embryonic cDNA library (Brown and Kafatos 1988). We were unsuccessful in isolating a cDNA clone by this approach, so we designed a reverse primer specific to the cloned *Dmyt1* fragment and used it in combination with a pNB vector primer to PCR amplify the 5' end of a cDNA sequence from the same library. The fragment obtained was cloned and sequenced and the information was used to identify two cDNA clones from the Berkeley *Drosophila* EST Project database (GH08848 and LD34963). These clones were both fully sequenced and found to include identical coding regions that show significant sequence similarities to human and *Xenopus* Myt1 within the predicted kinase domain (LD34963 is 20 bp longer at the 5' end, but the sequences are otherwise identical except for the length of the poly(A) tail at the 3' end). The complete molecular characterization of the *Dmyt1* gene will be presented elsewhere (Z. Jin, S. Rabinovitch and S. D. Campbell, unpublished results).

3.2.2 Generation of *Dwee1* and *Dmyt1* transgenic stocks

pUAST-Dwee1 and *pUAST-Dmyt1*

To synthesize *pUAST-Dwee1*, a 2.2-kb *Dwee1* cDNA fragment was excised from pBluescript SK(+) by *KpnI/NotI* digestion and subcloned into the pUAST vector using the same restriction sites (Brand and Perrimon 1993). *pUAST-Dmyt1* was constructed by cloning a 1.9-kb *Eco*RI/*Xho*I fragment that includes the entire *Dmyt1* cDNA from LD34963 and inserting it into the pUAST plasmid vector, also cut with the same restriction enzymes.

pUASp-Dwee1 and *pUASp-Dmyt1*

The 2.2-kb *KpnI/NotI* *Dwee1* cDNA fragment (as above) was inserted into the pUASp vector (Rørth 1998) cut with the same restriction enzymes. A PCR-amplified *Dmyt1* cDNA from the LD34963 clone containing *KpnI/NotI* linker restriction sites was cloned into the pUASp vector. This clone was then sequenced to establish that no new mutations were introduced during PCR amplification.

pGMR-Dwee1 and *pGMR-Dmyt1*

The *g*lass *m*ultimer *r*eporter plasmid (pGMR; (Hay *et al.* 1994) was cut with *HpaI* and *NotI*. The *Dwee1* and *Dmyt1* cDNAs were isolated from pUASp vector constructs by cutting with *KpnI*, blunting with T4 DNA polymerase, digestion with *NotI*, and then gel purification. Insert and vector were joined with T4 DNA ligase and the products verified by colony PCR. The transgene constructs were then injected into *y w Drosophila* embryos, using a Δ2-3-helper plasmid.

3.2.3 Scanning electron microscopy

Flies of the desired genotypes were collected several days after eclosion, fixed, dehydrated, and critical-point dried essentially as described in Sullivan *et al.* (2000). Critical-point-dried flies were then either imaged directly with a Philips (Cheshire, CT) ESEM (model XL30 ESEM ODP) or sputter-coated with gold and imaged with a Jeol (Tokyo) scanning electron microscope (SEM; model JSM-630FXV).

3.2.4 Transmission electron microscopy

Fly heads of the desired genotypes were collected, fixed, and dehydrated as described in Sullivan *et al.* (2000). Dehydrated heads were embedded in Spurr resin (Spurr 1969) with propylene oxide used as a transition solvent. Embedded heads were sectioned to 60 nm thickness with a Diatome diamond knife using a Reichert-Jung ultramicrotome (model ULTRACUT E). Sections were collected in water on copper grids, stained with uranyl acetate and lead citrate, and viewed on a Philips transmission electron microscope (TEM; model Morgagni 268). Images were collected with a Soft Imaging System digital camera (model Megaview II).

3.2.5 Immunochemistry

Imaginal discs were fixed in 4% formaldehyde in PBS for 30 min at room temperature. Following fixation, the peripodial membrane was removed from the eye discs using tungsten needles. After blocking in 10% normal goat serum (NGS) made with PBS + 0.1% Tween-20 (PBT), the fixed discs were washed three times for 5 min in PBT and incubated at 4° overnight in primary antibody (rabbit antiphosphohistone H3; Upstate Biochemicals) at 1/600 dilution in 10% NGS. Discs were then washed four times for 10 min in 5% skim milk in PBT and incubated in preabsorbed secondary antibody (goat anti-rabbit conjugated with FITC; Jackson Immunoresearch, West Grove, PA) at 1/1000 dilution. Stained discs were washed four times for 10 min in PBT, stained with Hoechst 33258, and washed again in PBT. Eye discs were then separated from the optic lobe and mounted in 80% glycerol. Images were obtained on a Zeiss (Thornwood, NY) Axioskop 2 microscope equipped with a Photometrics (Tucson, AZ) SenSys digital camera.

3.3 Results

3.3.1 Ectopic expression of *Dwee1* in developing imaginal tissues

To examine the consequences of overexpressing *Dwee1* and *Dmyt1* in different tissues, we generated transgenic lines that can express either gene under control of the Gal4/UAS system, as described in Materials and Methods (Brand and Perrimon 1993). Figure 3-1 shows the effect of Gal4-induced expression of *UAS-Dwee1* in various tissues (hereafter "UAS" refers to the UAST constructs). The *pannier-Gal4* (*pnr-Gal4*) and *apterous-Gal4* (*ap-Gal4*) drivers are each expressed in the developing dorsal thorax (Calleja *et al.* 1996). When either of these Gal4 drivers is combined with one copy of *UAS-Dwee1*, reduced numbers of sensory bristles are seen on the dorsal thorax, compared to wild type (Figure 3-1A, B, and D). Flies with *ap-Gal4*-driven

UAS-Dwee1 also have upturned wings, suggesting that the dorsal compartment of the wing is smaller than the ventral compartment, consistent with these cells undergoing fewer cell divisions (data not shown). When two copies of the *UAS-Dwee1* transgene are driven by either *ap-Gal4* or *pnr-Gal4*, the bristle effects are more extreme and the dorsal epidermis is distorted, indicating that the phenotypic effects are sensitive to gene dosage (Figure 3-1C and E). Combination of the *ap-Gal4* driver with two copies of *UAS-Dwee1* yields a nearly bald dorsal thorax accompanied by a severe reduction of the scutellum (Figure 3-1C). A more extreme phenotype is seen when the *pnr-Gal4* driver is combined with two copies of *UAS-Dwee1*, producing a furrowed thorax, as if the two halves have failed to fuse properly (Figure 3-1E). This observation suggests that fusion may require temporally or spatially regulated cell divisions that can be blocked by our overexpression system. In the wing, *UAS-Dwee1* combined with a wing-specific *sd-Gal4* driver line produces extensive scalloping of the wing margin (Figure 3-1F) and an additional copy of *UAS-Dwee1* (Figure 3-1G) also increases the severity of this mutant phenotype.

Ectopic *Dwee1* expression in the eye produces a rough eye phenotype (Figure 3-2). In Figure 3-2A and B, are controls showing a wild-type eye and an eye from a fly with a single copy of the *ninaE-Gal4* driver, respectively (Freeman 1996). When *UAS-Dwee1* is combined with the *ninaE-Gal4* driver, the eye facets are disorganized and frequent duplications of bristles are observed (Figure 3-2C). *ninaE-Gal4* overexpression of *Dmyt1* produced a similar phenotype (not shown). The *Dwee1* and *Dmyt1*-induced rough eye phenotypes suggested to us that we could use *Dwee1* or *Dmyt1* transgenic flies in an assay system for identifying negative or positive regulators of mitosis, as described below.

3.3.2 Genetic interactions with *GMR-Dwee1* and *GMR-Dmyt1*

The GMR overexpression vector uses a Glass transcription factor-binding enhancer to direct transgene expression posterior to the morphogenetic furrow (MF) in the developing eye (Hay *et al.* 1994). This single component system thus provides a convenient tool for rapidly testing genetic interactions. After cloning the cDNAs for each gene into this vector, we observed that *GMR-Dwee1* and *GMR-Dmyt1* transgenic lines each show dosage-sensitive rough eye phenotypes. In ~12 independent transgene lines examined for each construct, the *Dmyt1*-induced phenotypes are consistently stronger than the *Dwee1*-induced phenotypes, suggesting a stronger effect of Myt1 on eye development that is not attributable to chromosomal position effects (data not shown). In Figure 3-3B we show an adult eye from a fly carrying four copies of *GMR-Dmyt1*, compared with a wild-type control eye (Figure 3-3A). Posterior to the MF, the second mitotic wave (SMW) generates a pool of uncommitted cells for recruitment into the developing ommatidial preclusters (Wolff and Ready 1991). To test our assumption that the aberrant phenotypes we observe when Wee1 or Myt1 are overexpressed are a consequence of inhibiting or delaying cell divisions required for normal development, we examined mitotic activity in eye imaginal discs isolated from a *GMR-Dmyt1* transgenic strain. Figure 3-3C shows mitotic activity in a wild-type third larval instar eye disc, visualized by antibody staining for phosphohistone H3. In discs isolated from a *GMR-Dmyt1* transgenic line, mitoses in the SMW are both reduced in number and delayed (inferred from the increased distance of mitotic cells from cells of the "first mitotic wave"; Figure 3-3D) when compared to wild type. Mitoses ahead of the morphogenetic furrow (the first mitotic wave) are unaffected by *GMR-Dmyt1*, as expected since *GMR*-driven expression does not occur in this region of the disc (Hay *et al.* 1994). We also observed that the ommatidial preclusters in the *GMR-Dmyt1* flies appear disorganized when visualized by transmission electron microscopy of sectioned adult eyes. Figure 3-3E and F, shows the effects of *GMR-Dmyt1* on the arrangement of photoreceptor cells. Most of the identifiable cell types in the ommatidia appear to be present, although the arrangement and size of the rhabdomeres are often irregular. *The GMR-Dmyt1* photoreceptor cell clusters often contain too few or too many cells, however, and there is a striking disruption of the regular hexagonal array of secondary and tertiary pigment cells that normally forms an interface between adjacent ommatidia (compare Figure 3-3E and F).

We next tested for genetic interactions with a set of cell cycle regulatory mutants that are predicted to either have a direct regulatory interaction with *Dwee1* or *Dmyt1* or play an independent role in Cdk1 regulation. Mutations in factors that normally promote the onset of mitosis should enhance the *Dwee1* or *Dmyt1* overexpression phenotypes, whereas mutations in genes that function to delay mitosis should show the reverse effect. Figure 3-4 illustrates several such interactions. A single transgene copy of *GMR-Dmyt1* produces a mild rough eye phenotype, whereas independently, a heterozygous mutation in *cdc25*^{string} has no effect on eye morphology (Figure 3-4A and B). When a single copy of *GMR-Dmyt1* is combined with a heterozygous mutation for *cdc25*^{string}, a significantly enhanced eye phenotype is seen (Figure 3-4C). Likewise, removal of a single copy of *cdc2* produces a similar effect in combination with a single copy of *GMR-Dmyt1* (Figure 3-4D). The *GMR-Dmyt1/cdc25*^{string} interaction produces an eye that is devoid of bristles, whereas the *GMR-Dmyt1/cdc2* interaction shows milder bristle effects. Curiously, the dominant enhancement seen in these cases is consistently stronger in more anterior parts of the eye that differentiate later in development. Cdc2 (now called Cdk1) and its activating phosphatase, Cdc25^{string} are essential for promoting mitosis in *Drosophila* (Edgar and O'Farrell 1989; Stern *et al.* 1993), so these genetic interactions are consistent with known functions for these genes. A weak single-copy *GMR-Dwee1* phenotype (Figure 3-4E) is also enhanced by heterozygous mutant alleles of *cdc2* (Figure 3-4G), but unlike *GMR-Dmyt1*, not by heterozygous mutations for *cdc25*^{string} (not shown). These genetic interactions were confirmed with multiple alleles of *cdc2* and *cdc25*^{string} to rule out nonspecific genetic background effects. We also tested a number of other known cell cycle mutants for dominant modifier effects on either *GMR-*

Dwee1 or *GMR-Dmyt1* phenotypes. Mutations in *cyclin A*, *cyclin B*, *mei-41*, *grapes*, *twine*, *cdk2*, *cyclin E*, *fizzy*, and *dacapo* all fail to either enhance or suppress the rough eye phenotype generated by either transgene.

The *roughex* (*rux*) gene encodes a cyclin-dependent kinase inhibitor (CKI) that inhibits Cyclin A/Cdk1 by promoting the degradation of cyclin A (Thomas *et al.* 1994; Sprenger *et al.* 1997; Thomas *et al.* 1997; Foley *et al.* 1999; Avedisov *et al.* 2000). When *GMR-Dmyt1* (Figure 3-4I) or *GMR-Dwee1* (not shown) is coexpressed with *GMR-rux* the phenotype is enhanced relative to that generated by *GMR-rux* alone (Figure 3-4H), resulting in a stronger rough eye phenotype that is accompanied by a near complete loss of bristles. While this result is consistent with additive effects of these Cdk1 inhibitors, we also made the surprising observation that otherwise viable zygotic *Dwee1* mutants show near-complete synthetic lethality with otherwise viable zygotic rux mutants. Rare double-mutant "escapers" from these genetic crosses show various phenotypic abnormalities, including enhancement of the *rux* rough-eye phenotype, bristle duplications and deletions, and "Minute" bristles (data not shown).

To investigate genetic interactions with a known component of the DNA damage response pathway, we tested the *Drosophila* homologue of the *p53* tumor suppressor gene. Expression of a *p53-pExP-glass* transgene promotes apoptosis, generating eye tissue that has no evidence of intact ommatidia or bristles (Ollmann *et al.* 2000 ; Figure 3-4J). Coexpression of a single transgene copy of either *GMR-Dmyt1* (Figure 3-4K) or *GMR-Dwee1* (not shown) can markedly suppress this phenotype, with recovery of the eye bristles being most pronounced (compare Figure 3-4J with 3-4K).

The *tribbles* (*trbl*) gene encodes a novel mitotic inhibitor that functions in mesodermal cells during early gastrulation (Großhans and Wieschaus 2000; Mata *et al.* 2000; Ollmann *et al.* 2000; Seher and Leptin 2000). *ninaE-Gal4*-driven *UAS-Dwee1* or *UAS-trbl* transgenes alone generate slightly roughened eyes, with occasional duplication of bristles (Figure 3-5A and B). When the two genes are coexpressed in the eye, the ommatidial phenotype is dramatically enhanced and there is a near complete loss of bristles (Figure 3-5C). In a complementary experiment, the eye phenotype generated by two copies of *GMR-Dmyt1* combined with a single copy of *GMR-Dwee1* is partially suppressed by removal of one gene copy of *trbl* (data not shown). These striking synergistic interactions are not confined to eye development, as coexpression of *UAS-Dwee1* and *UAS-trbl* yields nearly complete ablation of wing tissue (Figure 3-5F), compared with scalloping of the wing margin observed when *UAS-Dwee1* or *UAS-trbl* are expressed singly with *the sd-Gal4* driver (Figure 3-5D and E). Occasional conversions of wing tissue to apparent thoracic tissue were also noted in these coexpression experiments. Unlike the similar wing margin phenotypes we observe when *UAS-trbl* or *UAS-Dwee1* are expressed during wing development, *UAS-trbl* expression is associated with a noticeable reduction of trichome density in the wing blade that apparently reflects increased cell size, a phenotype that is not observed with *UAS-Dwee1* (compare Figure 3-5D and E).

We next conducted genome-wide screens for loci that *modify GMR-Dwee1* or *GMR-Dmyt1* eye phenotypes, using the *Drosophila* deficiency kit (maintained by the Bloomington Drosophila Stock Center). The kit presently comprises 195 stocks that are estimated to cover 70–80% of the *Drosophila* euchromatic genome. In two separate screens, we tested these deletions for their ability to enhance the eye phenotypes associated with single-copy transgenic stocks of either *GMR-Dmyt1* or *GMR-Dwee1*. In a third screen to identify both enhancer and suppressor loci, we tested the deletions against a stock carrying two copies of *GMR-Dmyt1* and one copy of *GMR-Dwee1* (made by recombination of different transgene insertions). The genetic crosses were scored without reference to whether or not the deletions uncovered any known cell cycle regulators, to avoid biasing our results. The genetic loci that we have identified in these screens, as cytological regions defined either by deletions or by mutations in specific genes, are compiled in Table 3-1. Consistent with observations based on single alleles, *Df(2L)Mdh*, which includes the *cdc2* locus, enhances the phenotype of all three tester strains, whereas deletions that include *cdc25*^{string} [*Df(3R)3450* and *Df(3R)Dr-rv1*] were selected as enhancers of *GMR-Dmyt1* and *2xGMR-Dmyt1*, *1xGMR-Dwee1* in this assay, but not as enhancers of the *GMR-Dwee1* transgene alone.

Six deletions, four of which represent loci not previously identified in crosses with known cell cycle regulators, were identified as specific enhancers of *GMR-Dmyt1* (Table 3-1). One of the *GMR-Dmyt1* enhancer regions [*Df(3R)Dl-BX12*] contains *Delta* (*Dl*), which encodes a ligand for signaling through the Notch pathway. Independent tests with specific alleles of *Dl* have confirmed that *Dl* is the gene responsible for this interaction. Since some alleles of *Dl* exhibit dominant eye phenotypes (specifically, *Dl*^{1}), it is important to note that we observed enhancement with alleles (*Dl*^{3}, *Dl*^{7}, *Dl*^{B2}, and *Dl*^{RevF10}) that by themselves are not associated with a dominant eye phenotype. It is unlikely, therefore, that these interactions reflect additive effects. We saw similar enhancement with gene duplications of the *Notch* locus, which on their own are associated with a "Confluens" or *Delta*-like phenotype [*Dp(1;2)51b*, *Dp(1;2;Y)w*$^{+}$, and *Dp(1;2)72c21*]. A deletion of the *Notch* locus, on the other hand [*Df(1)N-8*], suppresses the phenotype associated with a *2xGMR-Dmyt1*, *1xGMR-Dwee1* strain. Specific genes responsible for the remaining three *GMR-Dmyt1* enhancer interactions have not yet been identified. *Df(2L)r10* contains three known mitotic regulatory genes (*grapes*, *twine*, and *fizzy*), none of which behaves as an enhancer in tests with specific mutant alleles, however. It is possible that the phenotypic modification seen with this deletion reflects a combinatorial interaction with more than one of these genes.

Only two cytological regions, identified by crosses to the deletion collection, were identified as specific enhancers of a *GMR-Dwee1* eye phenotype, one of which contains cdc2 (Table 3-1). We have not yet identified the gene responsible for the remaining suppressor interaction with *2xGMR-Dmyt1*, *1xGMR-Dwee1* that is associated with *Df(3L)st4*. Further analysis to identify and characterize the remaining gene modifiers will now be necessary to determine if these loci do in fact encode distinct regulators for *Dwee1* and *Dmyt1*.

3.4 Discussion

The G1/S and G2/M cell cycle transitions are temporally and spatially controlled during metazoan development, allowing growth and cell division to be coordinated with patterning and differentiation (reviewed by Edgar and Lehner 1996). Studies of G2/M checkpoint controls in metazoans have emphasized regulatory mechanisms affecting the Cdc25-like phosphatases, which activate the mitotic regulator Cdk1 by removing inhibitory phosphorylation. Regulatory mechanisms affecting the activity and protein stability of the Cdk1 inhibitory kinases are still poorly understood, but are probably just as important (Michael and Newport 1998; Lee *et al.* 2001). There are ample precedents for these mechanisms from studies of Wee1 and Mik1 kinases in *S. pombe* (Russell and Nurse 1987b; Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993; O'Connell *et al.* 1997; Raleigh and O'Connell 2000) and Swe1 in *S. cerevisiae* (Lew and Reed 1995; Sia *et al.* 1996; Sia *et al.* 1998; Barral *et al.* 1999; Edgington *et al.* 1999; McMillan *et al.* 1999).

During the third larval instar, the *Drosophila* eye disc undergoes progressive transformation from a relatively amorphous epithelial sac into the complex arrangement of ommatidial facets that comprises the adult compound eye. This transformation is marked by passage of a constriction called the MF across the eye disc (Wolff and Ready 1991). Cells within the MF normally arrest in G1 and failure to synchronize cells at this stage disrupts ommatidial patterning (Thomas *et al.* 1994). Following the MF, a population of cells called the SMW undergoes a final cell cycle. If cells are blocked in G1 by overexpression of a p21 CKI homologue, insufficient cells are left to form all of the cell types required for normal ommatidia, resulting in a rough adult eye phenotype (de Nooij and Hariharan 1995; de Nooij *et al.* 1996). In this report, we have shown that *GMR*-driven misexpression of *Dmyt1* immediately after the MF both delays the SMW divisions and reduces the numbers of mitotic cells, also resulting in a rough eye phenotype.

We have established that *Dwee1* and *Dmyt1* overexpression eye phenotypes are sensitive to modification by mutations in known cell cycle regulatory genes, illustrating the feasibility of screening for mutations of genes that are potential regulators of either Wee1 or Myt1. Mutations in genes that promote mitosis, such as *cdc2* and *cdc25*^{string}, should dominantly enhance these overexpression phenotypes and we have confirmed this expectation for both of these genes with *Dmyt1*. Although a *GMR-Dwee1* eye phenotype is also enhanced by mutations in *cdc2*, it is not enhanced by mutations in *cdc25*^{string}, providing evidence that Wee1 and Myt1 kinases have distinct Cdk1 regulatory effects in this developmental context. This result could be explained by a requirement for higher levels of *cdc25*^{string} activity to overcome *GMR-Dmyt1* inhibition of Cdk1 relative to *GMR-Dwee1*, perhaps because it is inherently more difficult to dephosphorylate Cdk1 inhibited on both T14 and Y15 by Myt1 activity, compared with Cdk1 inhibited on Y15 alone by Wee1.

The *rux* gene encodes a novel Cdk1 inhibitor that controls the onset of S phase during embryogenesis, eye development, and spermatogenesis (Gonczy *et al.* 1994; Thomas *et al.* 1994; Sprenger *et al.* 1997; Thomas *et al.* 1997; Foley *et al.* 1999; Avedisov *et al.* 2000). A recent study has shown that *rux* also plays a novel role in mitosis, by an unknown mechanism (Foley and Sprenger 2001). Rux and Wee1 both negatively regulate Cdk1 activity; thus our observation that coexpression of these genes generates more extreme rough eye phenotypes than seen with either alone is consistent with known functions for these genes. Surprisingly, we also found that flies lacking both zygotic *Dwee1* and *rux* functions show nearly complete synthetic lethality, with rare escapers exhibiting extensive adult bristle phenotypes. This interaction suggests that *rux* and *Dwee1* may also cooperate in some other, as yet undefined regulatory mechanism. The extensive bristle phenotypes seen in *rux* ; *Dwee1* double mutant escapers could indicate disruption of cell cycle timing or abrogation of genome integrity checkpoints, similar to the phenotypes seen in *mus304* mutants exposed to ionizing radiation, which are associated with increased genome instability (Brodsky *et al.* 2000). Another piece of evidence suggesting a role for Wee1 kinases in regulating genome stability is the interaction we observe with *Drosophila p53*. In humans, the p53 tumor suppressor promotes apoptosis in cells that have suffered DNA damage. Overexpression of *Drosophila p53* in the eye promotes extensive cell death by apoptosis, resulting in extremely defective eyes (Ollmann *et al.* 2000). We have shown significant suppression of the *p53* overexpression eye phenotype by coexpression of either *GMR-Dwee1* or *GMR-Dmyt1*, suggesting that these Cdk1 inhibitory kinases can negatively regulate p53-induced apoptosis. Since Cdk1 activity has previously been implicated in promoting apoptosis, this effect would be consistent with known functions of Wee1 and Myt1 in Cdk1 inhibition (Zhou *et al.* 1998). Other reports relevant to this issue are somewhat contradictory, however. In human cell culture, Wee1 can inhibit granzyme B-induced apoptosis; furthermore, Wee1 appears to be downregulated through a p53-dependent mechanism, suggesting that p53 regulation of Wee1 might normally occur during this process (Chen *et al.* 1995; Leach *et al.* 1998). In contrast, Smith *et al.* (2000) showed that Wee1 activity can actually promote apoptosis in a *Xenopus* oocyte extract system. Further studies are clearly needed to establish the physiological significance of any purported roles for Wee1 or Myt1 in regulating apoptosis, p53-dependent or otherwise.

A screen for modulators of *wee1* overexpression was previously conducted in *S. pombe*, by isolating suppressors of *wee1*-induced lethality (Aligue *et al.* 1994; Munoz *et al.* 1999; Munoz and Jimenez 1999). These studies identified mutations in the gene encoding the Hsp90 chaperone as potent suppressors, suggesting a role for Hsp90 in promoting the assembly and/or disassembly of functional Wee1 protein complexes. In contrast, we have not found *hsp83* mutant alleles (encoding Drosophila Hsp90) to act as suppressors of a combined *GMR-Dmyt1/GMR-Dwee1* transgene eye phenotype (data not shown). We have, however, identified several other genetic loci as specific enhancers of eye phenotypes generated by *GMR-Dwee1* or *GMR-Dmyt1* alone, indicating that phenotypic effects mediated by Wee1 and Myt1 are responsive to lowered expression of different genes. These observations may reflect differences in threshold requirements for the relevant gene products in promoting mitosis (as suggested by the interactions with *cdc25*^{string}) or they may signify differences in the regulation of Wee1 and Myt1 kinases that we will now be able to dissect by identifying and characterizing the relevant modifier loci. We are currently undertaking direct genetic screens for mutations in genes that modify *GMR-Dwee1* and *GMR-Myt1* eye phenotypes to address this issue. One of the loci we have identified as a specific enhancer of the *GMR-Dmyt1* eye phenotype is *Delta*. This interaction could reflect defects in *Dl*-dependent neuronal specification that are enhanced by *GMR-Dmyt1* activity, or it may indicate a novel role for Delta/Notch signaling in regulating Myt1 activity. We are presently trying to distinguish these possibilities.

In *S. pombe*, the DNA damage and DNA replication checkpoint pathways that regulate Cdk1 by inhibitory phosphorylation act by controlling the activity and stability of Wee1 and Mik1 kinases, as well as Cdc25 phosphatases (reviewed by Walworth 2000). Although metazoan homologs of components of these checkpoint pathways show significant sequence conservation with their yeast homologs, the actual functions and interactions of individual components are not necessarily conserved. For example Guo and Dunphy (2000) showed that *Xenopus* homologues of the checkpoint kinases Chk1 and Cds1, which respond to DNA damage and blocked DNA replication, respectively, in *S. pombe*, respond in the exact opposite manner to these stresses in *Xenopus* egg extracts. This example serves as a warning that simple predictions of metazoan gene function based on extrapolation from known functions of yeast genes can be misleading. Metazoan development requires that novel regulatory mechanisms exist to link specific developmental processes with the basic cell cycle machinery. *Drosophila* represents an ideal model for analyzing these developmental controls of the cell cycle, since the effects of specific mutations on complex processes like morphogenesis and differentiation can be established. The recent characterization of the *trbl* gene in *Drosophila* illustrates this point (Großhans and Wieschaus 2000; Mata *et al.* 2000; Seher and Leptin 2000). Trbl activity delays mitosis in invaginating G2 cells (mitotic domain 10) in a cycle 14 embryo. Although *cdc25*^{string} transcription initiates in domain 10 before it is transcribed in other cells, these cells remain G2 arrested until they are completely internalized, well after cells in nine other mitotic domains have subsequently expressed *cdc25*^{string} and entered mitosis (Edgar and O'Farrell 1989). Trbl activity downregulates Cdc25^{string} protein stability, providing an explanation for these observations (Mata *et al.* 2000). A similar purpose could be served by Trbl simultaneously upregulating *Dwee1* or *Dmyt1* activity (Großhans and Wieschaus 2000). Intriguingly, Trbl contains motifs reminiscent of Nim1-type kinases, which negatively regulate Wee1 and Swe1 kinase activity and stability in *S. pombe* and *S. cerevisiae* (Russell and Nurse 1987a; Coleman *et al.* 1993; Parker *et al.* 1993; Wu and Russell 1993; Barral *et al.* 1999). Despite these sequence similarities, the Trbl protein apparently lacks a functional catalytic domain, raising the possibility that Trbl could act in a "dominant negative" manner to activate Wee1 (or Myt1) by interfering with the activities of Nim1-like inhibitors. Genetic interactions that we describe in this report are consistent with this possibility.

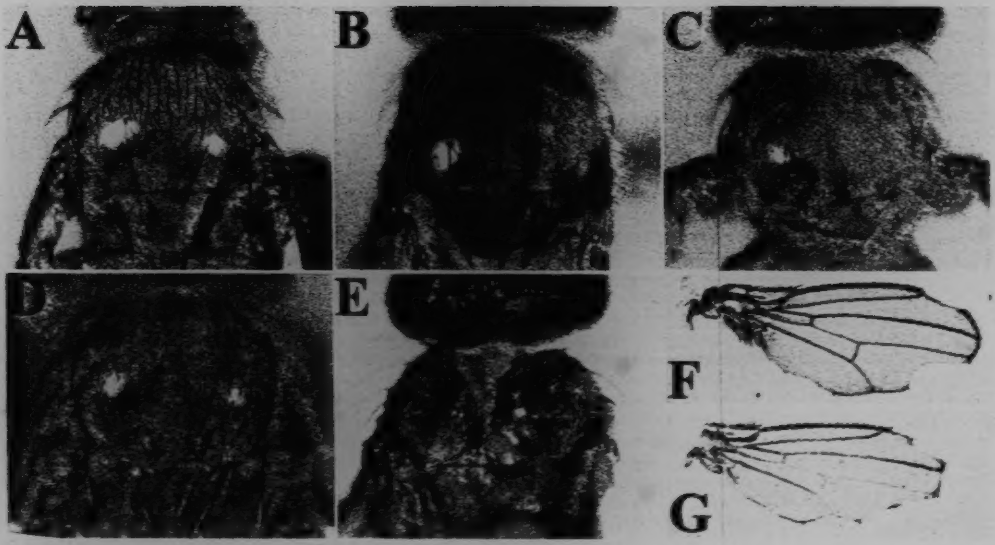


Figure 3-1
Aberrant phenotypes caused by *Dwee1* overexpression. (A) Thorax of a wild-type fly. (B) Thorax of a fly with a single copy of *UAS-Dwee1* driven by a single copy of *ap-Gal4*. (C) Thorax of a fly with two copies of *UAS-Dwee1* and a single copy of *ap-Gal4*. (D) Thorax of a fly with a single copy of *UAS-Dwee1* and a single copy of *pnr-Gal4*. (E) Thorax of a fly with two copies of *UAS-Dwee1* and a single copy of *pnr-Gal4*. (F) Wing of a fly with a single copy of *UAS-Dwee1* and a single copy of *sd-Gal4*. (G) Wing of a fly with two copies of *UAS-Dwee1* and a single copy of *sd-Gal4*.

Figure 3-2
Effects of *Dwee1* overexpression on the adult eye as visualized by SEM. (A) A single copy of the *ninaE-Gal4* driver transgene. (B) A single copy of the *UAS-Dwee1* transgene. (C) A single copy of *UAS-Dwee1* driven by a single copy of the *ninaE-Gal4* transgene.

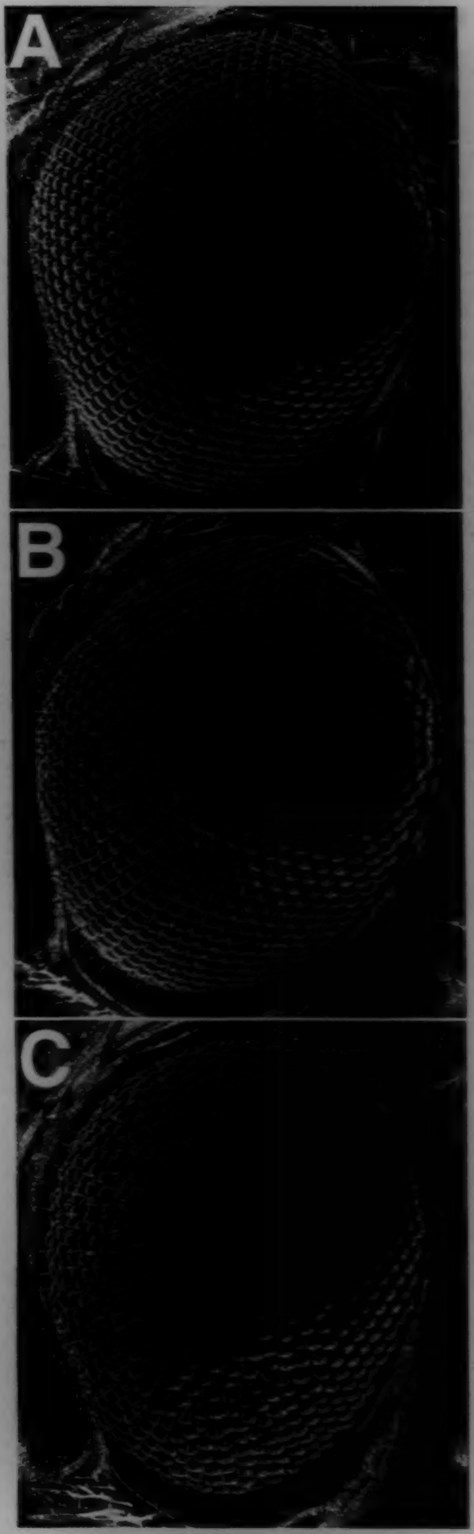
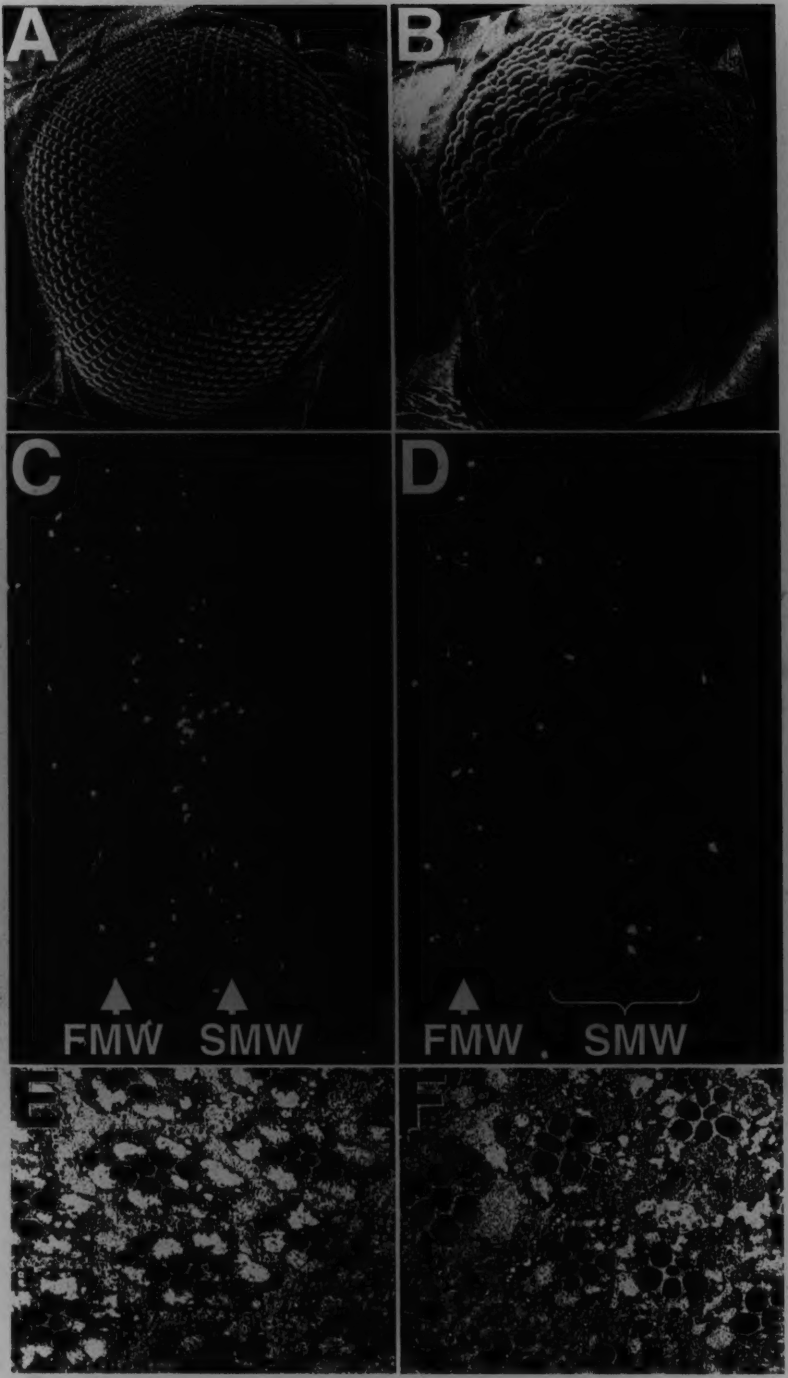


Figure 3-3
Effects of *Dmyt1* overexpression in the developing and adult eye. (A) SEM of an eye from a wild-type fly. (B) SEM of an eye from a fly with four copies of *GMR-Dmyt1*. (C) Eye-antennal disc from a wild-type fly stained with the mitotic marker, antiphosphohistone H3 (αPH3), showing mitotic figures in the first (FMW) and second (SMW) mitotic waves. (D) PH3-stained eye-antennal disc from a fly with four copies of *GMR-Dmyt1*. The SMW is disrupted and delayed, as shown by the decreased number and increased spread of mitotic figures posterior to the FMW. (E) TEM cross section of an adult eye from a wild-type fly. (F) TEM cross section of an adult eye from a fly with four copies of *GMR-Dmyt1*.



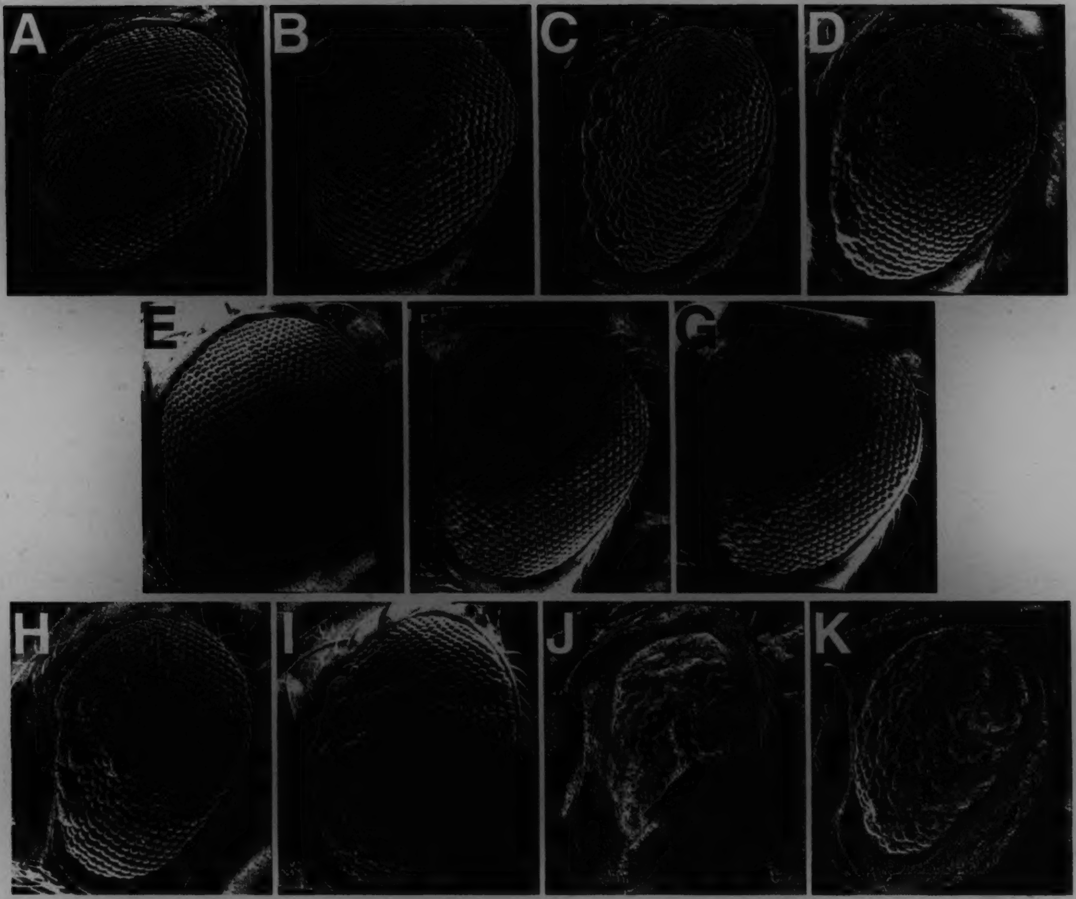


Figure 3-4
SEM analysis of eye phenotypes seen in genetic interactions with *GMR-Dwee1* and *GMR-Dmyt1*. (A) SEM showing a fly with a single copy of *GMR-Dmyt1*. (B) Fly heterozygous for a mutation in the *cdc25*^{string} locus. (C) Fly with a single copy of *GMR-Dmyt1* and heterozygous for a mutation in the *cdc25*^{string} locus. (D) Fly with a single copy of *GMR-Dmyt1* and heterozygous for a mutation in the *cdc2* locus. (E) Fly with a single copy of *GMR-Dwee1*. (F) Fly heterozygous for a mutation in the *cdc2* locus. (G) Fly with a single copy of *GMR-Dwee1* and heterozygous for a mutation in the *cdc2* locus. (H) Fly with a single copy of *GMR-rux*. (I) Fly with single copies of both *GMR-Dmyt1* and *GMR-rux*. (J) Fly with a single copy of *p53-pExP-glass*. (K) Fly with single copies of both *GMR-Dmyt1* and *p53-pExP-glass*.

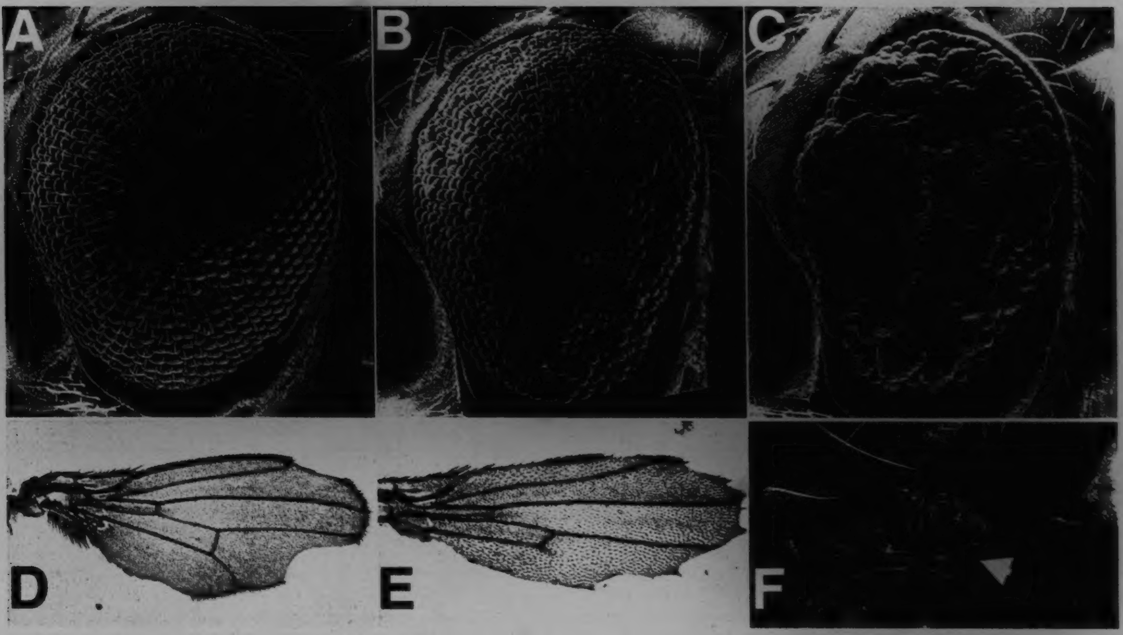


Figure 3-5
Coexpression of *Dwee1* and *trbl* shows strong synergistic phenotypic effects. (A) SEM of a fly with one copy of *UAS-Dwee1* driven by one copy of *ninaE-Gal4*. (B) Fly with one copy of *UAS-trbl* driven by one copy of *ninaE-Gal4*. (C) Fly with single copies of both *UAS-Dwee1* and *UAS-trbl* driven by a single copy of *ninaE-Gal4*. (D) Wing of a fly with one copy of *UAS-Dwee1* driven by *sd-Gal4* (hemizygous on the X chromosome). (E) Wing of a fly with one copy of *UAS-trbl* driven by *sd-Gal4*. (F) Fly with single copies of both *UAS-Dwee1* and *UAS-trbl* driven by *sd-Gal4*. The arrowhead indicates the position of the small piece of wing tissue.

Test Stock	Enhancement	Suppression
GMR-Dmyt1	*cdc2*	
	cdc25^{string}	
	Delta	
	Df(2L)net-PMF (021A01;021B07-08)	
	Df(2L)Mdh (030D-30F;031F)	
	Df(2L)r10 (035D01;036A06-07)	
	Df(3L)pbl-X1 (065F03;066B10)	
	Df(3R)Dl-BX12 (091F01-02;092D03-06)	
	Df(3R)3450 (098E03;099A06-08)	
	Df(3R)Dr-rv1 (099A01-02;099B06-11)	
GMR-Dwee1	*cdc2*	
	Df(2L)Mdh (030D-30F;031F)	
	Df(3R)e-R1 *(093B06-07;093D02)*	
2xGMR-Dmyt1, 1xGMR-Dwee1	*cdc2*	Notch
	cdc25^{string}	*tribbles*
	Delta	*Df(1)N-8* (003C02-03;003E03-04)
	Df(2L)net-PMF (021A01;021B07-08)	*Df(3L)st4* (072D10;073C01)
	Df(2L)Mdh (030D-30F;031F)	
	Df(2L)r10 (035D01;036A06-07)	
	Df(2R)vg-C (049A04-13;049E07-F01)	
	Df(3R)Dl-BX12 (091F01-02;092D03-06)	
	Df(3R)3450 (098E03;099A06-08)	
	Df(3R)Dr-rv1 (099A01-02;099B06-11)	

Table 3-1
Summary of interacting mutations/deficiencies.

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4 A Genetic Screen for Modifiers of Wee1-like Kinase Overexpression in *Drosophila* Implicates the Notch Pathway in Controlling Inhibitory Cdk1 Phosphorylation

4.1 Introduction

Cell cycle controls in unicellular eukaryotes respond to signals intrinsic and extrinsic to the individual cell. Intrinsic signals derive from sensing mechanisms which monitor cell mass, completion of metabolic processes such as DNA replication and repair, assembly of the cytoskeleton, chromosome alignment, and morphogenesis (Skibbens and Hieter 1998; Lew 2000; Murakami and Nurse 2000; Rua *et al.* 2001). Extrinsic signals include the sensing of nutrients, changes in osmolarity, and mating cues from surrounding cells (Fields 1990; Banuett 1998; Forsberg and Ljungdahl 2001). These mechanisms for sensing cell-extrinsic signals have been co-opted through multicellular evolution to convey growth inhibitory or proliferative signals in the elaboration of diverse tissues and cell types (Widmann *et al.* 1999; Schmelzle and Hall 2000). Cell growth, proliferation, death, and morphogenesis must be coordinated in the development of a multicellular organism–not only at the level of individual cells and within local cell populations, but between higher order cell populations and the organism they comprise.

Such controls are being widely studied in a variety of model systems, and these typically operate at the G1/S transition in the cell cycle. Cell-extrinsic signals acting at G2 in metazoans have been studied less extensively, however in recent years a number of developmental growth and proliferation controls acting at G2/M have been identified (Edgar and O'Farrell 1989; Edgar *et al.* 1994; Abrieu *et al.* 1997; Walter *et al.* 1997; Bitangcol *et al.* 1998; Fisher *et al.* 1998; Katzen *et al.* 1998; Murakami and Vande Woude 1998; Palmer *et al.* 1998; Walter *et al.* 2000; Ito *et al.* 2001; Okada *et al.* 2002; Okumura *et al.* 2002; Peter *et al.* 2002). These mechanisms transmit cell-extrinsic signal information by modifying the activity of the cyclin-dependent kinase Cdk1. Regulation of Cdk1 activity controls entry into mitosis and may be used to effect cell growth, proliferation, morphogenesis or death in a variety of developmental contexts.

The mitosis-promoting Cdk1 complex (comprised of a $p34^{cdc2}$-like protein kinase and a cyclin regulatory subunit) is subject to regulation through control of cyclin abundance, protein inhibitors and inhibitory phosphorylation. Kinases of the Wee1 family inhibit the Cdk1 complex (Featherstone and Russell 1991; Parker *et al.* 1992; Parker and Piwnica-Worms 1992; McGowan and Russell 1993); in metazoans these include the predominantly nuclear Wee1-like proteins, and cytoplasmic/membrane-bound Myt1-like proteins. The activities of Wee1-like kinases are countered by members of the Cdc25 family of mitosis-promoting protein phosphatases (Gautier *et al.* 1991; Strausfeld *et al.* 1991; Lee *et al.* 1992).

The Notch pathway is a widely conserved signal transduction mechanism employed throughout metazoan development for cell-cell communication. Notch signaling is used both cell-autonomously and non-autonomously to regulate cell proliferation and patterning, and cell-autonomously to specify cell fates (reviewed by Artavanis-Tsakonas *et al.* 1999). A number of recent studies have shown that Notch signaling can influence cell proliferation in *Drosophila* by modulating expression of the *cdc25*^{string} phosphatase (Johnston and Edgar 1998; Baonza and Garcia-Bellido 2000; Deng *et al.* 2001; Lopez-Schier and St Johnston 2001).

Here we describe a genetic screen to identify regulators of the Wee1-like kinases, Dwee1 and Dmyt1, in *Drosophila*. Our early findings indicate that alterations in Notch signaling modify Wee1-like kinase overexpression-induced eye phenotypes. This suggests a novel function for Notch in controlling cell proliferation via Wee kinase-induced inhibitory phosphorylation of Cdk1.

4.2 Materials and Methods

4.2.1 A screen for modifiers of *wee* kinase overexpression

Isogenous *w*; *dp/dp*; *e/e* males were starved and desiccated for 4 hours, fed on EMS (15-25mM in 1% sucrose solution) and mated *en masse* to virgin females carrying appropriate *w+*-marked *Dwee1* or *Dmyt1*-overexpressing GMR construct(s) on the 3rd chromosome (Price *et al.* 2002). These females also carried a compound X chromosome to permit recovery of X-linked mutations in F1 progeny males. Mutagenesis crosses were set up in batches of ~150 males and ~200 females per culture bottle. Each culture bottle was serially transferred onto fresh media for 5 days. F1 progeny males (exclusively) were scored for visible modification of the parental *GMR*-induced eye phenotype. Modifiers were assigned to a chromosome and stocked by simultaneously crossing F1 males to virgin female second (*y, w/y, w; Xa/CyO, dp*) and third chromosome (*y, w/y, w; Ly/TM6B, e*) balancer stocks. The marked balancer chromosomes allowed us to trace the inheritance of recessively marked mutagenized chromosomes. Modifiers were assigned to chromosomes as follows: crosses from which only *w+; dp/CyO, dp* progeny showed the modified phenotype were assigned to the second chromosome. Crosses that showed no modified progeny were assigned to the third chromosome (as the *w+*-marked *GMR*, also on the third chromosome, segregates away from the modifier in this case) and male *w-; e/TM6B, e* progeny were retained and tested by crossing back to the parental *GMR* stock. Balancer crosses which produced only modified female progeny were assigned to the X chromosome. Figure 4-1 shows a schematic diagram of the screening method.

4.2.2 Scanning Electron Microscopy

Flies of desired genotypes were collected several days after eclosion, fixed, dehydrated and critical-point dried essentially as described in (Sullivan *et al.* 2000). Critical point-dried flies were then imaged directly with a Philips ESEM (model XL30 ESEM ODP).

4.2.3 Analysis of potential transcription factor binding sites

Upstream sequences for *Dwee1*, *Dmyt1* and *cdc25^{string}* were obtained from Flybase (http://flybase.bio.indiana.edu/). The upstream sequence was pasted into the BioEdit DNA utility program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and defined sequence intervals selected (1000 or 2000 base-pairs). The obtained upstream sequence was then pasted into the 'TESS: Transcription Element Search Software on the WWW' service from the University of Pennsylvania (Schug and Overton 1997). Using the combined search page (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=SEA-FR-Query), upstream sequence was pasted into the "DNA Sequence" box. A high-stringency search of the 2000 base pairs upstream of each gene was performed to obtain the data shown in Table 4-2. A lower-stringency search covering only the 1000 base pairs upstream of each gene was performed to generate the data in table 4-3. For a more detailed description of the search procedure and the specific search parameters used, see the relevant section in the Appendix.

4.3 Results

4.3.1 Screens for modifiers of Wee1-like kinase overexpression

We conducted three separate F1 screens for dominant modifiers of Wee1-like kinase overexpression-induced eye phenotypes. These screens were as follows: 1) a screen for dominant enhancers of a weak *GMR-Dwee1* eye phenotype; 2) a screen for dominant enhancers of a weak *GMR-Dmyt1* eye phenotype, and 3) a screen for dominant suppressors of a stronger eye phenotype produced by a recombinant chromosome carrying two copies of *GMR-Dmyt1* and a single copy of *GMR-Dwee1*. A schematic outline of the screen is shown in Figure 4-1. Approximately 30,000 EMS-mutagenized haploid genomes were examined in each of the screens. We isolated many putative modifier loci in each of these screens, but the present discussion will deal with the two screens for enhancers of either *GMR-Dwee1* or *GMR-Dmyt1*, as the set of putative suppressors from the third screen remains largely uncharacterized. These comprise a set of 20 second chromosome and 30 third chromosome suppressors, which exist as balanced stocks, but which have not been confirmed by crossing back to the 2x*GMR-Dmyt1*, 1x*GMR-Dwee1* test stock or ordered into complementation groups.

The set of putative enhancer loci from the first (*GMR-Dwee1*) screen was narrowed to include only three alleles of *cdc2*. This is roughly consistent with our previous analysis of the Bloomington *Drosophila* Deficiency Kit, where a screen of 70-80% of the genome identified only *cdc2* and one other locus (at 30D-31F) as dosage-sensitive enhancers of the *GMR-Dwee1* phenotype (Price *et al.* 2002). The *GMR-Dwee1* enhancer locus at 30D-31F was quite possibly detected as a consequence of a multilocus-dependent interaction within the deficiency.

We recovered many more *GMR-Dmyt1* enhancer mutants, in agreement with our results from the deficiency kit, in which six separate loci were defined as good dosage sensitive modifiers (Price *et al.* 2002). We again recovered as modifiers mutant alleles of expected cell cycle regulators, including two alleles of *cdc2* and four alleles of *cdc25*^{string}. The first novel *GMR-Dmyt1* enhancer locus generated in this screen maps to the third chromosome, and through genetic complementation we have shown it to be an allele of *Delta* (Price *et al.* 2002). The identification of this locus was greatly facilitated by the dominant *Delta*-like wing phenotype (characterized by ectopic wing vein material, particularly near the ends of the veins). The recognition of *Delta* in the screen cued us to test for genetic interactions with other Notch signaling pathway mutants (Figure 4-2).

A loss-of-function mutation in *Delta* dominantly enhances the Wee1-like kinase overexpression phenotype generated by a recombinant chromosome bearing two copies of *GMR-Dmyt1* and one copy of *GMR-Dwee1* (compare Figure 4-2B and D). An additional genomic copy of the *Notch* locus also acts as an enhancer in this assay, bearing out the fact that heterozygous mutations in *Delta* and a duplication of *Notch* produce similar wing phenotypes. A gain-of-function *Notch* "abruptex" mutant (N^{Ax}) also shows enhancement in this assay (Figure 4-2H). The *Enhancer of Split* complex (*E(Spl)-C*) genes encode a family of transcription factors that serve as effectors of Notch signaling in activating Notch target genes (Delidakis and Artavanis-Tsakonas 1992; Knust *et al.* 1992; Schrons *et al.* 1992; Jennings *et al.* 1994). Notch signaling (in conjunction with the Suppressor of Hairless protein) activates expression of the *E(spl)-C*, turning on Notch target genes (Bailey and Posakony 1995; Schweisguth 1995). Lowering the genomic dose of *E(spl)-C* by one half should therefore decrease either the domain of Notch expressing cells, the intensity of Notch signaling or both. As shown in Figure 4-2J, a single copy loss-of-function mutation in the *E(spl)-C* causes suppression of the Wee1-like kinase phenotype. This interaction is also seen with a heterozygous loss-of function mutation in *Notch* itself, which suppresses the eye phenotype shown in Figure 4-2B (not shown).

From what is currently known about the roles of Notch signaling in eye development, these interactions are not at face value internally consistent. Mutations predicted to reduce Notch signal (*Delta*, *Notch*, *Enhancer of Split* and hyperploidy for *Notch*) do not all produce the same type of interaction in this assay. Furthermore, the N^{Ax} mutant, which has been shown to elevate Notch signal also shows enhancement, contradicting the observed interaction with *Delta*.

We are currently trying to identify several other *GMR-Dmyt1* enhancer loci that were detected in the screen. As a starting point we performed complementation analysis of the *GMR-Dmyt1* enhancer loci against second and third chromosome deficiencies which were previously identified as *GMR-Dmyt1* enhancers. Five of the set of twelve second chromosome enhancer mutants failed to complement two of the deficiencies; these fit into two complementation groups, with a single mutation failing to complement *Df(2R)vg-C* (enhanced phenotype shown in Figure 4-3J) and four mutations failing to complement *Df(2L)net-PMF* (enhanced phenotype shown in Figure 4-3H). Of these four *Df(2L)net-PMF* non-complementing *GMR-Dmyt1* enhancer mutations, three fail to complement for viability when crossed to each other, and the fourth gives a *Minute*-like adult bristle phenotype when placed *in trans* with the other three. Two of these alleles also show a *Delta*-like phenotype *in trans* with another of the enhancer-containing deficiencies, *Df(2L)r10* (enhanced phenotype shown in Figure 4-3D). We are in the process of obtaining all available lethal mutations known to map within *Df(2L)net-PMF* and *Df(2R)vg-C* to test them against the non-complementing *GMR-Dmyt1* enhancer mutants as a means of identifying the specific genes represented by these mutations.

Two additional third chromosome *GMR-Dmyt1* enhancer mutants fail to complement *Df(3R)Dl-BX12* (enhanced phenotype shown in Figure 4-3F) with a pupal lethal phenotype. These may represent atypical alleles of *Delta*. The complementation-mapped enhancer loci are summarized in Table 4-1. We are currently performing *inter se* complementation with all of the remaining loci which could not be assigned to any deficiency (seven on the second chromosome and twenty-two on the third). Given that the deficiency kit is estimated to cover only 70-80 per cent of the euchromatic genome, it remains possible that real enhancer loci were detected in the screen which do not fall within any of the deficiencies in the current version of the kit. Such enhancer loci will require subsequent meiotic mapping to narrow down their genomic location.

4.3.2 Analysis of the upstream regions of *Dwee1*, *Dmyt1* and *cdc25*^{string}

With the goal of identifying controls affecting the transcription of *Dwee1* and *Dmyt1* in development, I conducted a database search of upstream sequence from the *Dwee1*, *Dmyt1* and *cdc25*^{string} genes for conserved transcription factor binding sites. The searches were conducted with two different lengths of sequence at two stringency levels. The first set of potential transcription factor binding sites (diagrammed in Table 4-2) was obtained by searching the 2000 base pair upstream regions at a high statistical cutoff value (see the relevant section in the Appendix for the parameters used). This was done to identify high likelihood sites, and to generate a manageable number of candidates. The list is by no means a comprehensive summary of the returned results. Listed binding sites were selected on a subjective basis taking into account their potential involvement in the regulation of these genes in a cell cycle-specific manner, or in response to patterning gene inputs. The second set of binding sites shown in Table 4-3 was returned from a search with lower stringency cutoff values, using only the 1000 base pairs upstream. This shortened interval was used to generate a manageable number of sites with the decreased stringency threshold–the rationale being that a lower threshold value should permit detection of genuine sites which might conform less rigidly to a given consensus and would therefore be excluded from the first search. This second set of search results was manually scanned, and a subjective decision made about which of the many sites to include in the table.

The upstream sequence of the *cdc25*^{string} gene contains a vast expanse (~30 kb) of regulatory modules which integrate patterning gene inputs (Edgar *et al.* 1994; Lehman *et al.* 1999). Although a detailed analysis of the genomic region upstream of *cdc25*^{string} has not been published, the pattern of *cdc25*^{string} expression is disrupted in many patterning and segmentation gene mutants (Arora and Nusslein-Volhard 1992; Edgar *et al.* 1994; Johnston and Edgar 1998). The present analysis detected binding sites for many transcription factors encoded by the genes disrupted in these mutants, including Hunchback, Fushi tarazu, Twist, Dorsal, Kruppel, Even-skipped, Paired, Caudal, Snail and Zen (data not shown). Several of the same sites are also present in common in the *Dwee1* and *Dmyt1* upstream regions (Hunchback, Fushi tarazu, Paired, Snail, and Twist) with a Caudal binding site found separately upstream of *Dwee1* and Even-skipped and Kruppel sites upstream of *Dmyt1* (data not shown).

The Sp1 transcription factor participates cooperatively in the regulation of many cell cycle regulated genes (Fry and Farnham 1999), and its binding site is also found upstream of all three genes (Table 4-2). The expression of *cdc25*^{string} is also disrupted in mutants for *buttonhead*, which encodes an Sp1-like transcription factor (Edgar *et al.* 1994; Crozatier *et al.* 1996).

Binding sites for a number of cell cycle-regulated and checkpoint proteins were also found. p53 encodes a DNA damage-inducible transcription factor which promotes DNA repair, cell cycle arrest or apoptosis. A p53 binding site was found in the upstream region of *Dwee1*, but not *Dmyt1* or *cdc25*^{string}. p53 has previously been shown to negatively regulate *wee1* expression in human cells in response to DNA damage, and this promotes apoptosis (Leach *et al.* 1998). We have previously shown that overexpression of either *Dwee1* or *Dmyt1* via GMR in the developing eye imaginal disc suppresses a p53 overexpression phenotype, presumably due to blocking cell death (Price *et al.* 2002).

The AP-1 transcription factor consists of a heterodimer of Fos and Jun. Fos transcription takes place early after mitogenic stimulation, and is thought to play a role in cell cycle progression. A previous study has shown that the mouse *wee1* gene has an upstream AP-1 binding site, and its transcription is activated by AP-1 in response to antigen stimulation in mouse T cells (Kawasaki *et al.* 2001).

The E2F transcription factor is also a heterodimer, and is comprised of E2F and DP family subunits. E2F subunits serve to repress transcription of cell cycle and DNA replication genes in quiescent cells, and activating E2F subunits promote transcription of cell cycle and DNA replication factors in growth factor-stimulated cells. Previous studies have utilized DNA microarrays and chromatin immunoprecipitation to survey the human genome for E2F-regulated genes; these identified the CDC25C promoter, but not WEE1 (Ren *et al.* 2002; Weinmann *et al.* 2002). We observe E2F binding sites in all three promoters (Tables 4-2 and 4-3). In *Drosophila*, overexpression of the activating E2F in imaginal wing discs induces *cdc25*^{string} transcription (Neufeld *et al.* 1998). The failure of microarray-based studies to identify WEE1 as an E2F target may reflect the fact that these microarrays were not comprehensive of all human transcriptional units.

4.4 Discussion

Here I have presented a genetic screen for mutations that modify *Dwee1* or *Dmyt1* kinase overexpression phenotypes in *Drosophila*–most of which were identified as modifiers of a *Dmyt1* kinase overexpression phenotype. Loci which modify *Dwee1*- or *Dmyt1*-induced eye phenotypes are candidates for physiologically significant regulators of Wee1-like kinase expression, activity, or stability. This assay system can also detect regulators of G2/M that presumably are not controlling Wee1-like kinases, but have a direct role in G2/M progression (such as *cdc25*^{string} or *cdc2*). We have tested mutations in other potentially interacting cell cycle control factors quite extensively, and based on these tests the interactions we observe appear to be quite specific (Price *et al.* 2002). The fact that we isolated new mutant alleles of *cdc2* and *cdc25*^{string} in our screen, which we had previously shown interact with our test strains, validates our screening approach.

We have not yet characterized all of the material generated from the screen, but thus far a recurring theme has emerged and that is modification by Notch signaling pathway components. In *Drosophila*, *Notch* has been shown to encode a receptor whose activation is required for cell fate decisions (Heitzler and Simpson 1991; Rebay *et al.* 1991; Heitzler and Simpson 1993). *Delta* encodes one of its activating ligands. *Delta* and *Notch* were both identified as so-called neurogenic loci in screens for lethal mutations which produce neural hyperplasia (Lehmann *et al.* 1983). In *Drosophila* the Notch pathway operates at nearly every developmental stage, from early embryonic development, to development of larval structures, imaginal discs and pupal tissues (Artavanis-Tsakonas *et al.* 1999). Notch signals to define the boundaries of developmental patterning fields at a gross level (Irvine and Vogt 1997), but also as development progresses, to refine these gross patterning fields into specific structures such as individual neurons (Artavanis-Tsakonas *et al.* 1999).

In the differentiating eye imaginal disc, and in contrast with its classical neurogenic role, Delta/Notch signaling has an early proneural function–specifying the expression of the neuronal marker *atonal* in the three R8 equivalence group cells (Baker and Yu 1997; Baonza and Freeman 2001; Li and Baker 2001). This proneural role is independent of the *E(spl)-C*. Notch reverts to a neurogenic function later when the three cells of the R8 equivalence group are pared to one (Baker *et al.* 1996). Finally, in pupal development, Notch signaling is necessary to induce apoptosis in excess eye cells which are not part of any ommatidial cluster (Yu *et al.* 2002).

Since Delta/Notch signaling operates at multiple stages–and with differing outcomes–during eye development, the effect of modulations in Notch signal on Wee1 kinase overexpression phenotypes could potentially occur at any one or possibly more than one of several of these iterations of Notch signaling. In the present study we have driven Wee1-like kinase expression in a well-defined spatio-temporal pattern in the differentiating imaginal eye disc. The GMR vector drives transgene expression immediately posterior to (or behind) the morphogenetic furrow (MF) as it passes across the eye imaginal disc (Hay *et al.* 1994). *GMR*-driven transgene expression continues late into pupal development.

We have shown that *GMR*-driven Wee1-like kinase expression suppresses an eye phenotype produced by ectopic p53 (Price *et al.* 2002). This p53-induced eye phenotype is a manifestation of excess cell death (Ollmann *et al.* 2000), and the suppression we observe is presumably a result of hindering this cell death. While we have also shown that *GMR*-driven Wee kinase overexpression blocks cell division in the second mitotic wave (SMW), it is possible that the interaction we observe with Notch signaling is not a result of failure to generate sufficient cells in the nascent ommatidia, but rather exacerbation of reduced Notch-dependent apoptosis in the pupal eye. This seems especially likely since none of the previously described roles for Notch signaling at this stage of eye development is implicated directly in controlling cell proliferation.

The eye can tolerate reduced or excessive numbers of cells with remarkably little change in its morphology. The relative contribution to the observed Notch interaction of blocking the SMW mitosis (generating too few cells early) versus a subsequent effect in offsetting Notch-dependent cell death (preserving too many cells late) could be ascertained by treatments which re-create these two conditions in isolation. *GMR*-driven expression of the $p21^{CIP1/WAF1}$ CKI blocks the SMW with only marginal effect on gross eye morphology. Likewise, *GMR*-driven expression of the anti-apoptotic factor p35 blocks at least three rounds of cell-death in the imaginal and pupal eye disc, and produces a relatively intact eye despite a vast excess of undifferentiated cells (Hay *et al.* 1994). Differential interaction of either treatment in mutants for the Notch signaling pathway should indicate which of these potential roles is the relevant one in producing the interactions we observe with Notch.

The inconsistency of the interactions we observe is perplexing, but is not unprecedented in studies examining interactions with Notch. Given the multiple signaling iterations, *E(spl)-C* dependent and independent signals, and the fact that loss or gain of Notch signaling can generate similar phenotypes, it is no surprise that straightforward interpretations are not forthcoming.

Epidermal growth factor (EGF) signaling from nascent ommatidial preclusters posterior to the MF produces the cell division of the SMW in surrounding cells by inducing their expression of $cdc25^{string}$ (Baker and Yu 2001). This generates a pool of cells which are then recruited into the developing preclusters to fulfill the necessary complement of specialized cell fates (Wolff and Ready 1991; de Nooij and Hariharan 1995). The mechanism by which EGF signaling induces *cdc25*^{string} expression is unknown. Since *GMR*-driven *Dmyt1* blocks the SMW, presumably by countering the effect of EGF-induced *cdc25*^{string} expression, we anticipated that EGF signaling might coordinately down-regulate any or all of *Dwee1* and/or *Dmyt1* expression, activity, or stability. We have been unable to produce an interaction with hypomorphic EGFR alleles to back up this hypothesis, however (not shown).

The involvement of Notch signaling in controlling cell proliferation via modulation of Cdk1 inhibitory phosphorylation by Cdc25^{string} has been a recently documented phenomenon, and appears to operate at several developmental stages and tissues studied thus far. The directly upstream signal(s) by which Notch signaling regulates Cdc25^{string} expression (and possibly activity and stability) in these cases remain largely unknown, however. In the wing imaginal disc, *cdc25*^{string} transcription is indirectly repressed by Wingless via Achaete and Scute, possibly by their direct binding to the *cdc25*^{string} promoter (Johnston and Edgar 1998). *cdc25*^{string} transcription is indirectly activated by Notch in the dorsal and ventral regions immediately flanking the Dorsal/Ventral (D/V) boundary of the anterior compartment. Activated Notch signaling in these domains serves to inhibit expression of Achaete and Scute. A direct role for Notch signaling in promoting cell proliferation (that is, independent of its margin-inducing activity) has also been shown, but it remains unknown at this point how this might operate (Baonza and Garcia-Bellido 2000). Deng *et al.* (2001) and Lopez-Schier and St Johnston (2001) have shown that Notch signaling from the germline to the soma is required in *Drosophila* oogenesis to terminate the proliferation of follicle cells and their entry into endocycles. This is accomplished at least in part through transcriptional down-regulation of *cdc25*^{string}.

The *cdc25*^{string} gene has an enormous and complex upstream sequence, which contains binding sites for numerous segmentation gene products and homeobox-type transcription factors (Edgar *et al.* 1994; Lehman *et al.* 1999). Furthermore, the pattern of *cdc25*^{string} expression is altered in many patterning gene mutants (Edgar *et al.* 1994). This appears to represent an important mechanism by which cell proliferation and pattern formation are coordinated, although disrupting the pattern of cell division is well tolerated in the embryo (Edgar and O'Farrell 1990). The promoter regions of the Wee1-like kinases have not been characterized, but it seems likely given their directly opposing functions, that they might be regulated by the same factors, to opposite effect. Our analysis of upstream sequences is consistent with this hypothesis. Presumably cell type-specific transcriptional accessory molecules ensure that transcription of *cdc25*^{string} and the Wee1-like kinases is not activated simultaneously, since they carry out directly opposing functions.

It is also conceivable that corresponding regulation of Wee1-like kinases by the Notch pathway is *not* required in any of the developmental paradigms where it has been observed thus far for *cdc25*^{string}. In theory, a baseline level of Wee1-like kinase-catalyzed inhibitory phosphorylation of Cdk1 could be overcome by Notch-mediated activation of Cdc25^{string}, which through its activation of Cdk1 may indirectly cause the inactivation or degradation of Wee1-like kinases.

Interacting factors which act as transcriptional modifiers of *Dmyt1* would obviously have to act through their effect on the endogenous gene, as the GMR construct lacks any native regulatory sequences. For example, an enhancer mutation could act by ablating an inhibitor of *Dmyt1* transcription, raising the transcription levels of the endogenous gene. The analysis we have conducted thus far of potential transcription factor binding sites in the *Dmyt1* and *Dwee1* promoters yields no obvious clue to candidate mechanisms linking the expression of these genes to either Notch or EGF signaling (i.e., binding sites for transcription factors regulated by either of these pathways). The *Dmyt1* enhancer deficiency *Df(2L)net-PMF* contains the *net* gene, which encodes a transcriptional repressor of the Serum Response Factor family. Net is a transcriptional repressor of *rhomboid* in the intervein regions during wing development, and loss of net produces an expansion of rhomboid and appearance of ectopic veins (Brentrup *et al.* 2000). All existing *net* alleles are viable, while our candidate mutants in this region are lethal, furthermore, *net* has not been shown to play a role in eye development, however this might simply be an artifact of selections for *net* mutants which were based on wing phenotypes.

These candidate promoter-binding factors may prove informative in helping us identify enhancer mutants from the screen. Proof that any of these factors bind in the predicted fashion could be obtained through chromatin immunoprecipitation or electrophoretic mobility shift experiments. Promoter analysis through mutagenesis of these binding sites could reveal important insights into the regulation of these kinases in development. Since it appears likely that *Dwee1* and *Dmyt1* have redundant functions, this may require simultaneous disruption of both genes.

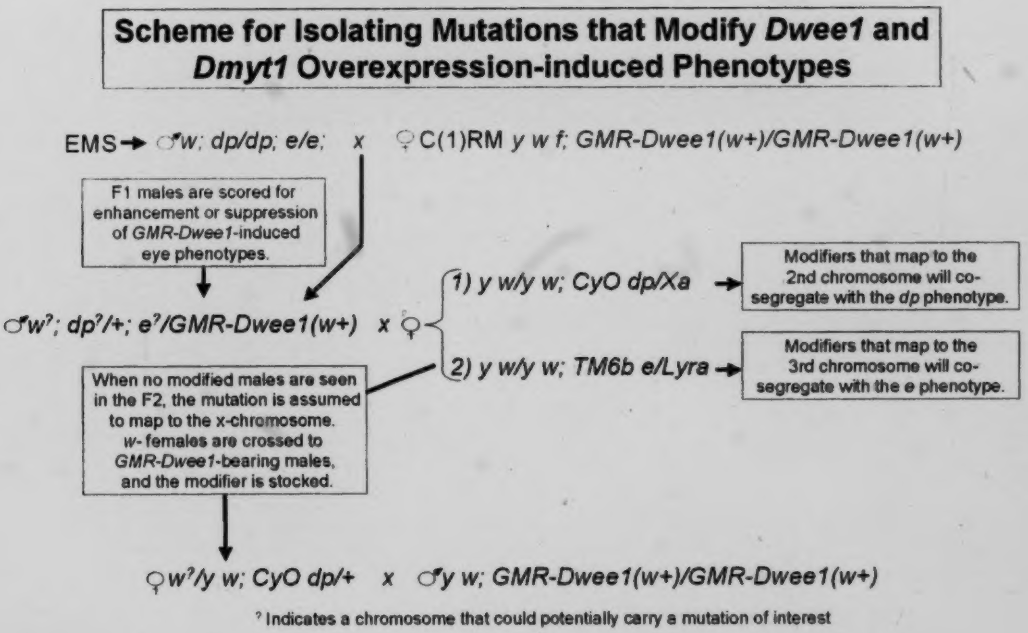
Scheme for Isolating Mutations that Modify Dwee1 and Dmyt1 Overexpression-induced Phenotypes
EMS → ♂w; dp/dp; e/e; x ♀C(1)RM y w f; GMR-Dwee1(w+)/GMR-Dwee1(w+)
F1 males are scored for enhancement or suppression of GMR-Dwee1-induced eye phenotypes.
♂$w^{?}$; $dp^{?}$/+; $e^{?}$/GMR-Dwee1(w+) x ♀
1) y w/y w; CyO dp/Xa
Modifiers that map to the 2nd chromosome will co-segregate with the dp phenotype.
2) y w/y w; TM6b e/Lyra
Modifiers that map to the 3rd chromosome will co-segregate with the e phenotype.
When no modified males are seen in the F2, the mutation is assumed to map to the x-chromosome. w- females are crossed to GMR-Dwee1-bearing males, and the modifier is stocked.
♀$w^{?}$/y w; CyO dp/+ x ♂y w; GMR-Dwee1(w+)/GMR-Dwee1(w+)
? Indicates a chromosome that could potentially carry a mutation of interest

Figure 4-1
Scheme for isolating mutations which modify Wee1-like kinase overexpression-induced eye phenotypes. The diagram shows the outline for enhancers of *GMR-Dwee1*, but the same scheme is followed for the screens for enhancers of *GMR-Dmyt1* and suppressors of *2xGMR-Dmyt1, 1xGMR-Dwee1* (discussed in greater detail in the Materials and Methods section of the text).

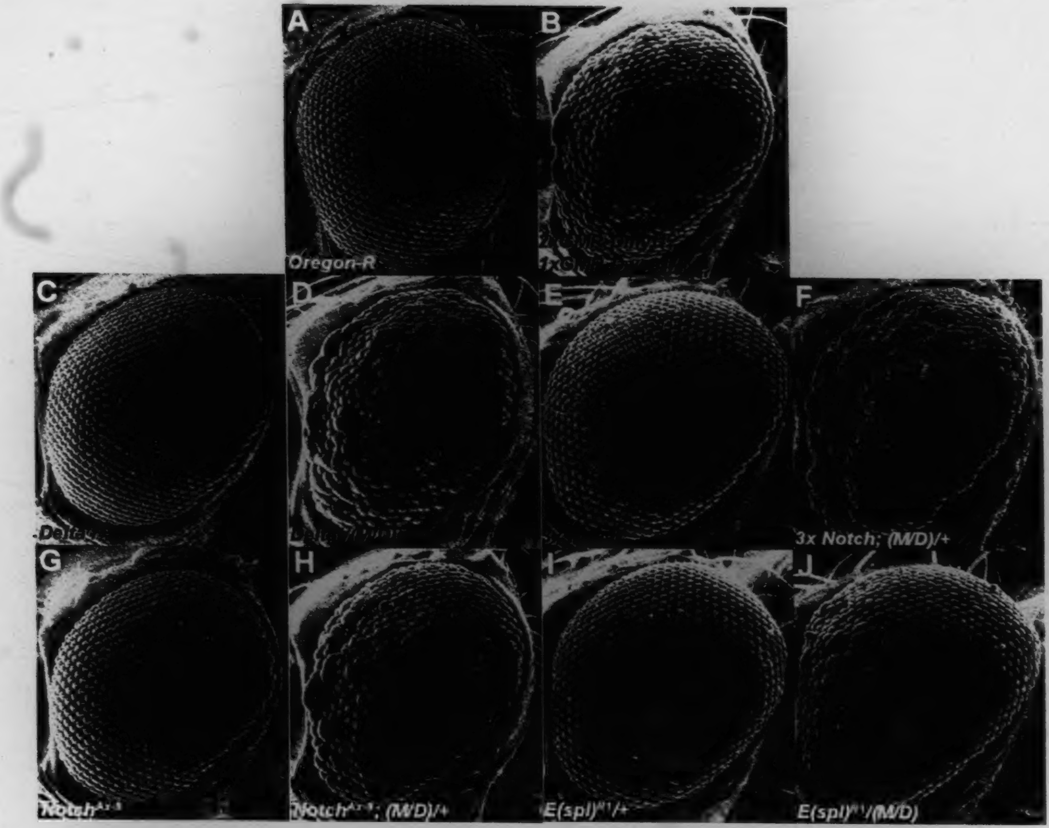

Figure 4-2

Alterations in Notch signaling modify a Wee1-like kinase overexpression-induced eye phenotype. (A) and (B) are comparative controls as in the previous figure. The genotype designation shown in (B) is abbreviated to (M/D) in the proceeding panels. (C) and (D) Mutation in the Notch ligand-encoding *Delta* gene dominantly enhances the combined *GMR-Dmyt1/GMR-Dwee1* phenotype. (E) and (F) Additional gene dosage of the *Notch* locus also enhances this phenotype. (G) and (H) An activated *Notch* signaling mutant (N^{ax}) enhances this phenotype. (I) and (J) A loss-of-function mutation for the *Enhancer of split* Complex suppresses the Wee1-like kinase overexpression phenotype.

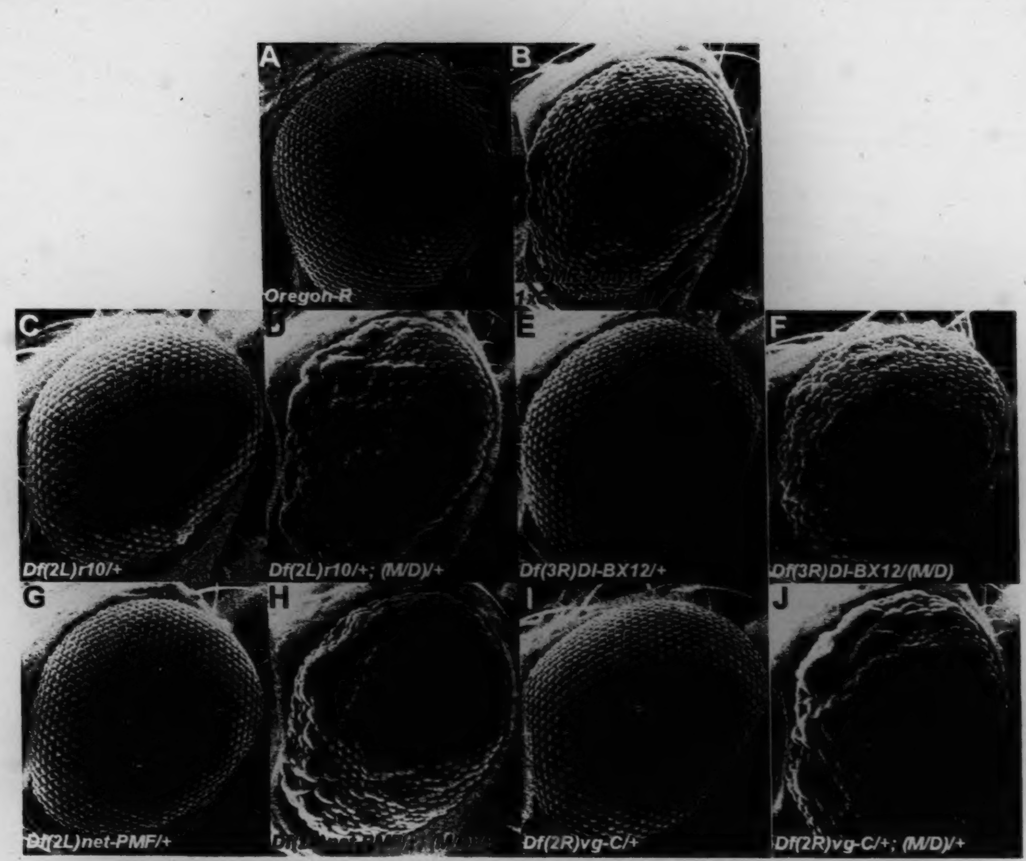

Figure 4-3
Deficiencies which enhance a Wee1-like kinase overexpression-induced eye phenotype. (A) Control eye from an adult wild type fly (strain Oregon-R). This is used for comparison with the phenotypes of deficiency stocks in the following panels. (B) Control eye from a fly carrying three GMR transgenes: two copies of *GMR-Dmyt1* and one copy of *GMR-Dwee1* (abbreviated to "(M/D)" in the remaining panels). This is used as the baseline for comparing the enhanced phenotypes in the succeeding panels. (C) and (D) Dominant enhancement of the phenotype in (B) by the deficiency *Df(2L)r10*. We have isolated a *GMR-Dmyt1* enhancer-bearing chromosome that fails to complement this deficiency (see the Results section). (E) and (F) Dominant enhancement of the phenotype in (B) by the deficiency *Df(3R)Dl-BX12*. We isolated an allele of *Delta* as an enhancer of *GMR-Dmyt1*, and it fails to complement this deficiency (see the Results section). (G) and (H) Dominant enhancement of the phenotype in (B) by the deficiency *Df(2L)net-PMF*. We isolated at least three alleles of a *GMR-Dmyt1* enhancer and these chromosomes fail to complement this deficiency (see the Results section). (I) and (J) Dominant enhancement of the phenotype in (B) by the deficiency *Df(2R)vg-C*.

Enhancer deficiencies	Non-complementing mutants
Df(2L)net-PMF	*ME33, ME34, ME38, ME41*
Df(2R)vg-C	*ME37*
Df(2L)r10	*Delta*-like ϕ over *ME34, ME41*
Df(3R)Dl-BX12	*Delta (ME30), ME7, M10, ME14,*

Table 4-1
Complementation results of second and third chromosome *GMR-Dmyt1* enhancer loci. These mutants are designated "ME" for *GMR-Dmyt1* enhancer.

Factor	*Dwee1*	*Dmyt1*	*cdc25*^{string}
AP-1	(-32) CTGACTGA		(-553) AGCAAACA
	(-319)TGACATCA		(-572) ATGATGTCAA
E2F			(-42) TTTGGCGC
			(-671) CGCGGCAAA
			(-820) GTTCGCGGCAAA
			(-1794) TTTCGCGC
MCM1/SRF/ TCF	(-730) ACTAATATGG	(-1532) CCTAATAAGT	
	(-757) CCTTAGATGG	(-1794) CCATTTATGA	
Net			(-324) GCAGGTAGTG
			(-992) GCAGGAAGAG
SP1	(-888) AGCTCCGGCCC	(-429) GTGCCGCCCA	(-375) GTGGAGGGGGC
	(-988) TGGGCGGCGA	(-479) TATTATGCCCTTA	(-381) GGGGCGTGGC
		(-913) GAGGCTGAGC	(1780) TGGGCGGTCC
		(-1732) AGGGAGGAGC	
		(-1978) CGGGCTGGTT	

Table 4-2
Highly conserved transcription factor binding sites identified in the 2000 bases upstream of the *Dwee1*, *Dmyt1* and *cdc25*^{string} genes.

Factor	*Dwee1*	*Dmyt1*	*cdc25*^{string}
p53	(-149) AGACTTGTT		
E2F	(-171) AGCCCGCCAA (-174) CCGCCAAA (-989) TGGGCGGCGA	(-559) ACGGCGCCTA	
Myc	(-802) TTACACGTGAGG		(-556) CAAACACGTGGAA
Myb		(-437) CCAACTGCG (-502) TTTAACGGT	
AP-1		(-699) TTACTAGGCGG	
Net	(-55) CTGGAAGT	(-62) AAATCCTG	

Table 4-3
Survey of moderately well conserved transcription factor binding sites identified in the 1000 bases upstream of the *Dwee1*, *Dmyt1* and *cdc25*^{string} genes.

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5 General Discussion and Conclusions

5.1 Overview

In the preceding chapters I have presented genetic analyses of a Wee1 kinase in *Drosophila*. This study represents an important advance in our understanding of the role played by this class of kinases in a developing metazoan organism. I also created an overexpression system for generating Wee1-like kinase overexpression-induced eye phenotypes, which can be used to assay for genetic interactions with candidate mutations or transgenes. This system was utilized in a genetic screen for modifiers of these eye phenotypes, which should include *bona fide* regulators of Wee1 kinases in development. The following is a general discussion of the results obtained in the course of this Ph.D. project, their implications for our thinking about Wee1 kinase function, and further studies to extend the results presented here.

5.2 *Dwee1* controls mitotic timing in the syncytial embryo

In this work I have shown that *Dwee1* is required maternally in the embryo to enforce proper timing of the late syncytial divisions (see the Appendix), and for successful completion of syncytial development (Price *et al.* 2000). A prior model offers a convenient prediction of how Dwee1 might act to control mitotic timing in the syncytial embryo (Sibon *et al.* 1997). This model states that a mitotic oscillator, presumably driven by the degradation of cyclins at mitosis, times the rapid divisions of the earliest cycles (1-8). Eventually the near logarithmic duplication of nuclei in the syncytium depletes maternally supplied factors to the extent that they become limiting for S phase, and DNA replication can no longer be completed in less time than the period of the mitotic oscillator. A checkpoint mechanism is now required to restrain the mitotic oscillator prior to receipt of a signal indicating that DNA replication is complete. The length of time that this checkpoint mechanism must postpone mitosis is increased with each cycle thereafter until cellularization. The rational target for this DNA replication checkpoint is Cdk1, whose periodic activation initiates the mitotic program (Edgar *et al.* 1994). There are a number of (potentially non-exclusive) ways in which Cdk1 activity might be restrained by this checkpoint, but inhibitory phosphorylation is the most obvious candidate mechanism given the class of mutants in which checkpoint function is abrogated (Sibon *et al.* 1997; Sibon *et al.* 1999; Brodsky *et al.* 2000; this work).

While this model is attractive, and seems to easily explain the phenomenology of mutant phenotypes (assuming a conventional function of Dwee1 in delaying mitosis via inhibitory Y15 Cdk1 phosphorylation), there is no compelling evidence at this point that the assumption it rests on is valid. Inhibitory Y15 Cdk1 phosphorylation has not been detectable in syncytial *Drosophila* embryos (Edgar *et al.* 1994). It remains possible that an undetected but nonetheless physiologically significant, fraction of Y15 inhibited Cdk1 exists and that its loss is responsible for the checkpoint defects observed in *Dwee1* mutant-derived embryos. This cryptic Y15 inhibited Cdk1 might be sequestered to a small subcellular compartment (such as the nucleus (Heald *et al.* 1993), however evidence for such compartmentalization has not yet been obtained (E. Homola, personal communication). Recently this laboratory has been able to detect a small amount of Y15 Cdk1 phosphorylation in syncytial embryo extracts, that is *not* eradicated in *Dwee1*-derived embryos, suggesting a further complication in how we view the events underlying mitotic catastrophe in the mutants (E. Homola, personal communication).

If Y15 inhibitory Cdk1 phosphorylation is not the mechanism by which maternal Dwee1 controls mitotic timing in the late syncytial embryo, what alternative mechanisms are there? At least two precedents exist for non catalytic Cdk1 inhibition by Wee1-like kinases (Liu *et al.* 1999; McMillan *et al.* 1999; Wells *et al.* 1999). If a catalysis-independent mechanism accounts for the Dwee1-dependent checkpoint, a kinase-null mutant of *Dwee1* should suffice to rescue the syncytial defect in *Dwee1*-derived embryos. However the tools for driving gene expression in the *Drosophila* germline are relatively crude. Re-creation of the pattern and levels of endogenous *Dwee1* expression sufficient to achieve rescue may ultimately require the development of transgenes which employ its native promoter and upstream sequences. We were successful in rescuing the maternal defect of *Dwee1* mutants by heat shock expression of a *Dwee1* transgene, but have been unable to generate rescue, or even detectable protein from a similar transgene construct which expresses a kinase null version of *Dwee1* (This study, unpublished, and E. Homola and S. Campbell, personal communication). This could indicate a problem with the expression or stability of this abnormal protein. Alternative ideas for how Dwee1 regulates entry into mitosis include the possibility that Dwee1 has another phosphorylation target, or some other completely novel enzymatic activity, either of which could be required for enforcing the *Dwee1*-dependent checkpoint. This last option must be considered speculative, as no published precedent for it exists.

5.3 *Dwee1* is inessential for zygotic development

The most unexpected result from this project is the observation that Dwee1 kinase function is dispensable for zygotically controlled development in the fly. Following upon this observation, several alternative possibilities present themselves:

1) Inhibitory Y15 phosphorylation of Cdk1 is not essential for viability. This possibility seems remote, although at this time it cannot be formally excluded. Cdk1 variants refractory to inhibitory phosphorylation have been expressed in a number of systems (Gould and Nurse 1989; Norbury *et al.* 1991), and this is generally deleterious if not fatal (except for the notable case of *S. cerevisiae,* where only specific checkpoint functions are impaired; Amon *et al.* 1992; Sorger and Murray 1992; Lew and Reed 1995). Non-

inhibitable Cdk1 has been expressed in *Drosophila* embryos, with lethal consequences (Lane *et al.* 2000). However this form of Cdk1 (Cdk1AF) does not permit specific examination of the role of, or requirement for, Cdk1 Y15 inhibitory phosphorylation, since both T14 and Y15 inhibitory sites are blocked–this experiment only establishes that one or both sites are essential for completion of embryonic development. To assay the role of each inhibitory phosphorylation event in isolation, independent variants will need to be constructed which ablate the respective inhibitory sites separately.

2) Residual maternal product can rescue the zygotic function of this gene by catalyzing inhibitory Cdk1 Y15 phosphorylation throughout development. This also appears unlikely, although precedents exist for similar types of maternal rescue of zygotic gene function in *Drosophila*. Females homozygous for the hypomorphic *Dwee1*^{DS1} mutant allele produce infrequent viable progeny, however a small fraction of embryos produced by these mutant females reach adulthood and can be scored. Crossing *Dwee1*^{DS1}/*Dwee1*^{DS1} females to *Dwee1*^{ES1}/+ males yields a preponderance of *Dwee1*^{DS1}/+ versus *Dwee1*^{DS1}/*Dwee1*^{ES1} progeny (see Appendix). If *Dwee1* was completely nonessential zygotically, equal numbers of both classes should be expected. This is consistent with our observations from other crosses–with heterozygous mothers–that generate *Dwee1* mutant flies, which show a distinct effect of crowding on viability (see below). There are no apparent phenotypic abnormalities in the *Dwee1*^{DS1}/*Dwee1*^{ES1} class produced in this cross. Since the *Dwee1*^{ES1} allele is null, if not slightly antimorphic (Price *et al.* 2000), one would expect complete absence of the *Dwee1*^{DS1}/*Dwee1*^{ES1} class from this cross if maternal protein was required to rescue an essential zygotic function.

3) There exists a redundant kinase which catalyses essential zygotic Y15 Cdk1 phosphorylation. This is the most likely explanation for the zygotic dispensability of *Dwee1*. While it remains to be shown that Y15 phosphorylation levels or patterns are unaltered in *Dwee1* mutant animals, this lab and another have identified a Myt1 kinase in *Drosophila* (Cornwell *et al.* 2002; Price *et al.* 2002), which in other systems has been shown to phosphorylate both the T14 and Y15 sites on Cdk1 (Mueller *et al.* 1995; Liu *et al.* 1997). If Dmyt1 has an overlapping role with Dwee1 in catalyzing Y15 Cdk1 phosphorylation, this could presumably account for the superfluity of either gene for zygotic viability (Price *et al.* 2000; Z. Jin, personal communication). The synthetic lethality of *Dwee1/rux* double mutants indicates that these two gene products function

redundantly in delaying mitosis. It remains to be seen if this interaction arises specifically from an inhibitory phosphorylation deficit. Additionally, whether the same phenomenon is observed with the *Dmyt1/rux* double mutant. It is also possible that some aspects of zygotic Y15 Cdk1 phosphorylation are disrupted in *Dwee1* mutants, but that disruption is tolerated or compensated for in some way. While inhibitory phosphorylation of Cdk1 is essential for embryonic development, its dysregulation is remarkably well tolerated. When Cdc25^{string} is expressed from a heat shock-inducible transgene in the cellular blastoderm embryo, it drives all cells into a simultaneous mitosis, presumably via removal of inhibitory Cdk1 phosphorylation (Edgar and O'Farrell 1990). This represents a complete derangement of the normally complex pattern of divisions in the mitotic domains (Foe and Alberts 1983; Foe 1989), but is nonetheless something from which embryos can recover, even after multiple rounds of synchronous Cdc25^{string}-driven mitosis (Edgar and O'Farrell 1990). It is therefore possible that the Y15 Cdk1 phosphorylation deficit is not completely compensated by Dmyt1 in all developmental stages and cell types in the *Dwee1* mutants. This may produce subtle differences that are not readily apparent under optimal laboratory conditions. *Dwee1* mutants are sensitive to crowding, and this could stem from one or both of two factors: these mutants may be more sensitive than their heterozygous siblings to elevated concentrations of metabolic waste products in the medium and simply fail at some stage in development. Or alternatively, the *Dwee1* mutants may be slightly retarded in coordination or locomotory responses, and are trampled into the medium by their siblings and die. Either or both of these scenarios are possible. Behavioral changes may be a good candidate for a zygotic deficit in *Dwee1* mutants, as the central nervous system undergoes extensive cell division after the rest of the (non-imaginal) larval tissues have ceased dividing. These late divisions may be particularly susceptible to the loss of Cdk1 inhibitory phosphorylation, and phenotypes generated by their disruption might be discernible only at the level of behavior. Well-defined gravitaxia, phototaxia and chemotaxia assays have been developed (Benzer 1973), and these could readily be used to test such locomotor responses in *Dwee1* mutant flies relative to their siblings.

5.4 *Dwee1* mutants are sensitive to hydroxyurea and caffeine

While not essential for viability, and not showing any overt phenotypic abnormalities, zygotic *Dwee1* mutants show a differential sensitivity to certain chemical agents. Hydroxyurea (HU; Price *et al.* 2000) and caffeine (Radcliffe *et al.* 2002, in press) are two chemicals tested thus far to which *Dwee1* mutants are sensitive. Both are likely to have relatively pleiotropic effects, but–and presumably of greatest relevance to the current analysis–HU has been shown to inhibit the enzymatic activity of Ribonucleotide Reductase (RNR; Elford 1968), and caffeine to inhibit the activity of Rad3/Ataxia Telangiectasia-Mutated (ATM) kinase homologues (Blasina *et al.* 1999; Sarkaria *et al.* 1999; Moser *et al.* 2000; Zhou *et al.* 2000). Blocking the activity of RNR interferes with DNA replication by depleting the pool of deoxyribonucleoside triphosphates (dNTPs), molecules that are the building blocks of DNA (Snyder 1984; Bianchi *et al.* 1986). The ATM-like kinases are known to be required for multiple checkpoint responses, but simultaneous loss of both branches (ATM and ATR) must disrupt important developmental cell division signals.

Dwee1 mutants also show moderate sensitivity to ionizing radiation (see Appendix) and the chemotherapeutic drugs cisplatin and ara-C, but not the alkylating mutagen methyl methanesulfonate (Radcliffe *et al.*, in press). Inasmuch as *Dwee1* and *Dmyt1* are likely to have both unique and redundant roles during development, it is possible that this relationship will also be maintained with respect to their functions in DNA checkpoints. The ATM-related kinases, ATM and ATR, show differential roles in the cellular response to various DNA damaging treatments and inhibitors of DNA replication. This may be reflected in their differential use of downstream signaling effectors (such as Chk1 vs.Cds1/Chk2, or Dwee1 vs. Dmyt1). Assuming that their combined activities are responsible for the sum total of Cdk1 inhibitory phosphorylation in the cell, it may therefore be expected that Dmyt1 would have a more significant role in the DNA damage response than Dwee1, or possibly in response to specific types of DNA damage, but any redundant role for these kinases in DNA checkpoints is likely to be masked by their (anticipated) combined requirement for viability.

5.5 *Dwee1* and *Dmyt1* do not produce equivalent phenotypes when overexpressed, nor do they respond to the same sets of interactors

GMR-Dwee1 and *GMR-Dmyt1* transgenes are not equivalent in their ability to induce phenotypes in the eye. This appears not to be simply a matter of position-effect on the transgenes under analysis, as multiple lines were isolated for both constructs, and the *GMR-Dwee1* phenotypes were consistently weaker than those for *GMR-Dmyt1*. While it is impossible to rule out a consistent difference in the stability or expression levels of the respective transcripts (for example, due to the nature of the UTR elements), the differences we observed with two transgenes in producing an interaction with mutations in *cdc2* vs. $cdc25^{string}$ (a *GMR-Dmyt1* phenotype is enhanced by single copy loss-of-function for either gene, while a *GMR-Dwee1* phenotype shows enhancement with only *cdc2*) may hint at a real difference in the regulation of *Dmyt1* and *Dwee1* and/or in the effects of their catalysis. Possibly there is a more potent effect of increased T14 phosphorylation, or the combination of T14 and Y15 phosphorylation catalyzed by Dmyt1 vs. the single Y15 phosphorylation catalyzed by Dwee1. This may account for both the differences seen in phenotypic severity, as well as differential sensitivity to modification. Additionally, it is possible that if there are specific cyclin/Cdk isoforms differentially targeted by Dwee1 vs. Dmyt1, those targeted by Dmyt1 may play a more significant role at this particular stage in eye development.

5.6 The *GMR-Dmyt1* overexpression phenotype is modified by alterations in Notch signaling

We noted interactions of the *GMR-Dmyt1* phenotype with alterations in Notch signaling. The observed interaction with Notch could conceivably operate at one or multiple levels of regulation. While a characterization of *Dmyt1* transcript or protein levels in various backgrounds of Notch signaling might prove insightful to the mechanism behind the *Dmyt1/Notch* pathway interactions, the ultimate test of the interaction between *Myt1* and the Notch signaling pathway will be to demonstrate a change in the readout of Myt1 activity. This could take the form of an increase in its activity commensurate with low levels of Notch signaling, and decreased levels with high Notch activity. E. Homola in our laboratory has developed a T14 phospho Cdk1-specific antibody to complement the commercially available Y15 phospho Cdk1-specific antibody. The Y15 phospho Cdk1 antibody has been used to detect Y15 inhibitory Cdk1 phosphorylation *in situ* in *Drosophila* (E. Homola, personal communication). A robust anti-*Dmyt1* antibody which could be used to visualize the protein *in situ* has not yet been generated, however transgenic lines expressing GFP-tagged versions have been produced (Z. Jin, personal communication).

5.7 Analysis of upstream sequences indicates potential differences in the transcriptional regulation of *Dwee1* vs. *Dmyt1*

In comparing the promoter regions of *Dwee1*, *Dmyt1* and *cdc25^{string}*, we identified transcriptional regulatory sites similar to those that have been characterized in other systems, as well as potentially novel sites. Notably, these three genes share some of the same conserved sites, raising the possibility that they are coordinately regulated at the level of transcription. This would be in keeping with their apparently coordinate regulation at the protein level by factors like Chk1/Cds1 and 14-3-3 proteins. Presumably cell type-specific accessory factors or chromatin conformation ensure that the effect on transcriptional activity of binding to these sites is reciprocal, as simultaneous transcription of both types of genes would be tantamount to driving with one foot on the brake and the other on the throttle.

While identification of a highly conserved sequence may be indicative of a mechanistic role for a given transcription factor in regulating a gene of interest, sequence conservation alone should not be taken as confirmation of a particular regulatory interaction. To confirm a regulatory interaction implied

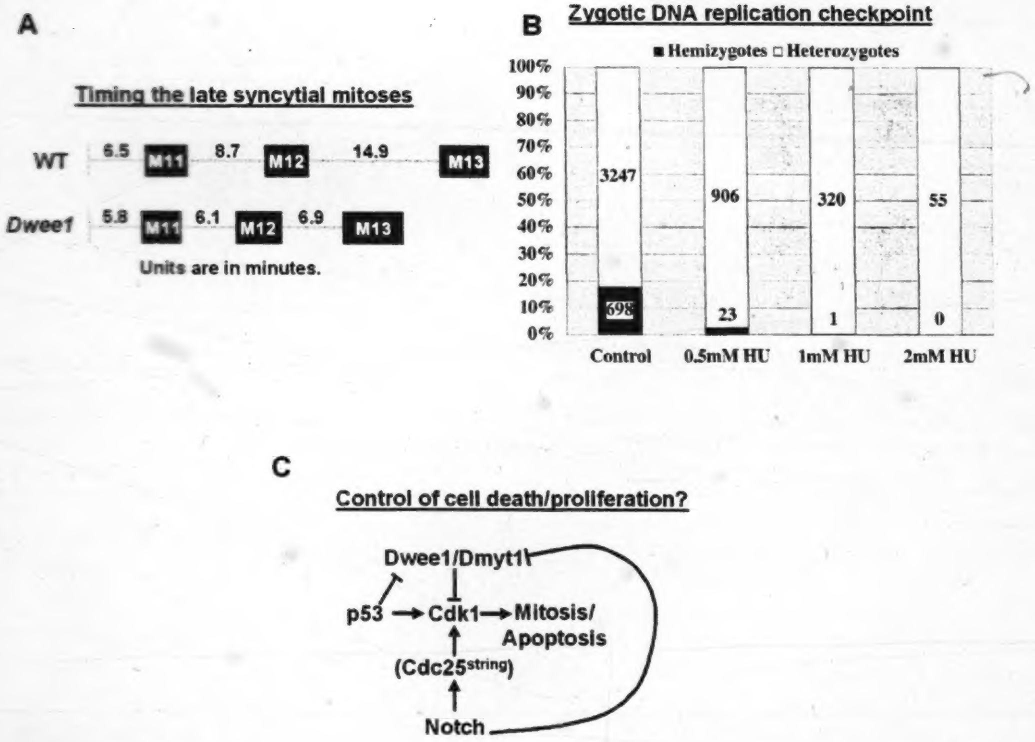

Figure 5-1
Basic summary of results presented in this thesis. (A) *Dwee1* controls the timing of the late syncytial mitoses. (B) *Dwee1* is dispensable zygotically, but *Dwee1* mutants are sensitive to hydroxyurea (HU), an inhibitor of DNA replication. (C) Model of interactions based on the results presented in the text.

from sequence analysis, a recently developed technique called chromatin immunoprecipitation (or ChIP) has proven invaluable. If a transcription factor binding site has been identified upstream of a gene, this interaction may be confirmed by fixing cells, sonicating them, extracting the DNA and bound protein, and immunoprecipitating the extract with antisera to the transcription factor in question. PCR primers flanking the predicted binding site are used to amplify the sequence, and if the transcription factor does bind to the predicted target, this DNA will be pulled down in the immunoprecipitation and a PCR product obtained. Once a transcription factor-binding site has been confirmed in this manner, the binding sequence may be deleted or modified to eliminate factor binding. The effect of this mutation may be confirmed *in vitro* by electrophoretic mobility shift assay (EMSA). The ultimate test of the *in vivo* role of this transcription factor binding would be to engineer a genomic rescue construct with this binding site ablated. The consequence of losing this aspect of regulation could then be assayed by its effect on the development of the fly. In the case of *Dwee1*, this rescue analysis would probably need to be performed in a double *Dwee1 Dmyt1* mutant background, assuming that this combination is required to achieve lethality. Alternatively, ablation of a transcriptional repressor site may be sufficient to generate developmental abnormalities in an otherwise wild-type fly, through ectopic expression of the protein.

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6 Appendix

Included in this appendix are experimental data not presented in the papers, and detailed descriptions of experimental methodology where appropriate.

6.1 Heat shock rescue of the maternal *Dwee1* phenotype

Collecting, fixing and mounting embryos

Embryos were collected from the surface of the food vial that they had been laid on overnight using distilled water. These embryos were washed in distilled water, and then dechorionated in 50% bleach in distilled water for two minutes. Dechorionated embryos were permeabilized in heptane for approximately 30 seconds, and then transferred in heptane to an eppendorf tube that contained 500ml of 37% formaldehyde fixative (formalin; Sigma), to a total volume of 1ml. Embryos were fixed for approximately 3 minutes and then the aqueous formaldehyde (bottom) phase was removed and replaced with 500ml of methanol. The tube was then shaken vigorously by hand for 1 minute. The heptane phase, embryos which failed to sink to the bottom of the tube, and residual methanol were discarded. The remaining embryos were washed in fresh methanol 3 times. Embryo collections were serially fixed in this manner daily, and stored in methanol at –20°C until the course of the experiment was complete. All of the stockpiled embryos were then re-hydrated by serial addition of PBT (1x phosphate-buffered saline with of 0.1% Tween-20; 25%, 50%, 75% and 100%). Re-hydrated embryos were stained with Hoechst 33258 (at 1 µg/ml) for 4 minutes and then washed 3x5 minutes with PBT. Stained embryos were mounted on glass slides in 80% glycerol and cover slips were placed on top. Cover slips were sealed with nail polish.

Scoring the rescue

After initially attempting to score a large collection of embryos by scanning back and forth across the slide, I found it was too easy to lose track of scored vs. non-scored embryos, so for the sake of accuracy I devised the following scoring method. Each mounted collection of embryos was photographed under ultraviolet light at 50x magnification. The entire field of embryos on the slide was photographed piecemeal, and a composite rendering of the all the embryos was spliced together into one large TIFF image in Adobe Photoshop. I went back through the embryos at 100x magnification, and using the composite TIFF as a reference, scored each embryo as Unfertilized (U), Arrested (A), Pre-arrest (P) or Rescued (R). Embryos were scored as "U" if only a single pronucleus was evident. Embryos were scored as "A" if they displayed fusion of nuclei with no evidence of cellularization. Embryos were scored as "P" if they were fertilized, but too early in development to be informative. Embryos were scored as "R" if they showed any sign of cellularization. The appropriate letter designation listed above was inscribed on the picture of each embryo in the composite image.

6.2 Determining mitotic timing in *Dwee1*-derived embryos

To assess the mitotic timing in *Dwee1*-derived embryos vs. wild type, Hemizygous *Dwee1* mutants were generated with a GFP-tagged histone transgene on the third chromosome. Embryos were collected in cages on grape juice agar plates streaked with yeast paste. Plates were changed several times each day when collections were not being made. Cages were changed and washed approximately every two days. Embryos were collected and hand-dechorionated by rolling onto double-stick tape with fine forceps. About 30 embryos were dechorionated and mounted onto cover slips streaked with a glue made from double stick tape dissolved in heptane. The mounted embryos were covered in heavy halocarbon oil to prevent desiccation, and were transported to the Biological Sciences Microscopy Service Unit. Movies were obtained on a Molecular Dynamics inverted confocal microscope. Images were collected at 200x magnification, and frames were collected once every minute, with a five second scan duration. Laser intensity was set at 10%. TIFF images were compiled into animations using GIFbuilder (http://homepage.mac.com/piguet/GifBuilder-1.0.sit.hqx). Data was compiled by going through the animations frame-by-frame and scoring the length of recognizable cell cycle intervals. Chromosome condensation was used as the indicator of mitosis. Nuclei were scored as being in interphase if their chromosomes were diffuse, and in mitosis at the first sign of condensation.

Data for Wild type embryos scored:

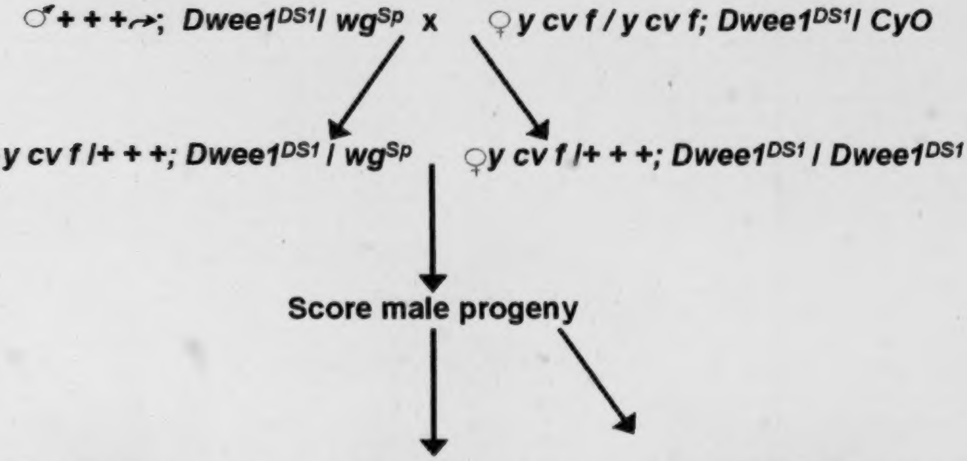
Stage	**WT34**	**WT35**	**WT39**	**WT40**	**WT41**	**WT42**	**WT43**	**Mean**
M10				5				**5**
I11		6	7	6	7			**6.5**
M11	5	6	5	4	5			**5.75**
I12	8	8	11	8	9	8	9	**8.714**
M12	5	6	5	5	5	5	5	**5.143**
I13	12	14	21	13	15	15	14	**14.86**
M13	6	6		6	5	6	6	**5.75**

Data for *Dwee1*-derived embryos scored:

Stage	**DW100**	**DW102**	**DW103**	**DW105**	**DW107**	**DW108**	**DW110**	**DW111**	**DW112**	**DW114**	**DW116**	**Mean**
M10											4	**4**
I11		6					6			6	5	**5.75**
M11		5		4		5	5	5	5	4	5	**4.75**
I12	6	6		6	7	7	6	6	6	6	5	**6.1**
M12	6	5	5	5	5	6	5	6	6	5	6	**5.455**
I13	7	7	7	7	8	7	7	6	7	7	6	**6.909**
M13	7	8	7	6	7	8	7		7	7		**7.111**

M=mitosis; I=interphase. Numbers indicate cycle number (e.g. M10=mitosis, cycle 10). Interval times are in minutes.

6.3 Scoring meiotic recombination in the *$Dwee1^{DS1}$* mutant


♂+ + +/↗; $Dwee1^{DS1}$/ wg^{Sp} x
♀y cv f / y cv f; $Dwee1^{DS1}$/ CyO
♀y cv f /+ + +; $Dwee1^{DS1}$ / wg^{Sp}
♀y cv f /+ + +; $Dwee1^{DS1}$ / $Dwee1^{DS1}$
Score male progeny

From FlyBase:	*$Dwee1^{DS1}$/ wg^{Sp}*	*$Dwee1^{DS1}$/$Dwee1^{DS1}$*
y-cv: 13.7cM	y-cv: 17.7cM	y-cv: 12.0cM
cv-f: 43.0cM	cv-f: 42.1cM	cv-f: 41.6cM
y-f: ~50.0cM	y-f: 52.9	y-f: 47.8cM
Combined: 56.7cM	Combined: 59.8cM	Combined: 53.6cM

A multiply marked x chromosome (*yellow crossveinless forked; y cv f*) was used to determine meiotic recombination frequency in *$Dwee1^{DS1}$* homozygotes versus heterozygotes. The progeny sample from the *$Dwee1^{DS1}$ /wg^{Sp}* mothers contained 1421 flies. The progeny sample from the *$Dwee1^{DS1}$ /$Dwee1^{DS1}$* mothers contained 1059 flies. Test mothers were crossed as virgins to Oregon-R males (a wild type strain). The *wg^{Sp}* marker was simply used to differentiate the *$Dwee1^{DS1}$* homozygotes and heterozygotes in this cross.

6.4 Assaying for ϒ radiation sensitivity of *Dwee1* mutants

Progeny from a cross producing *Dwee1*^{ES1}/*Df(2L)Dwee1*^{W05} hemizygous progeny as a %33 predicted fraction of the total progeny were treated with ϒ radiation at the indicated doses during the second larval instar period and surviving adult progeny were scored:

♂ *Dwee1*^{ES1} / *CyO* x ☿ *Df(2L)Dwee1*^{W05} / *CyO*

↓

ϒ radiation dose (Rad)	% hemizygous progeny	N
0	26.6	1235
500	30.7	688
1500	12.9	675
2000	15.4	149

A Co^{60} ϒ ray source ("Gammacell 220"–manufactured by Atomic Energy of Canada, Ltd.) was used to administer radiation doses. *Drosophila* culture vials were placed in the chamber, and at this position, based on the posted rates of decay, the dose was approximately equivalent to 100 Rads/minute. The experiment was performed in August to October of 1999.

The ϒ ray source was situated in the Chemistry Department at the University of Alberta at the time this experiment was performed, but has since been moved to the Alberta Research Council facility in Mill Woods. This relocation prevented the experiment from being as thorough as might have been desired, notably in the small sample size of the final dose class, which showed significant pupal lethality. I subjected flies to a further dose of 3000 Rads, but this produced lethality of all progeny from this cross.

1.5 Summary of crosses performed with GMR stocks and the Bloomington deficiency kit

The following table summarizes the interactions observed from crossing the GMR stocks described in the text to the Bloomington *Drosophila* Deficiency Kit. Deficiency stocks are listed in the order of their cytological location. This list with hyperlinks to more detailed information on each stock may be found on the World Wide Web at: http://flystocks.bio.indiana.edu/df-kit.htm.

Chromosome	Stock #	Cytological Interval:	*GMR-Dmyt1*	*GMR-Dwee1*	*2xGMR-Dmyt1, 1xGMR-Dwee1*	Potential Interactors
DK1	1329	001A01;002A	-	-	-	
DK1	1546	001B02-14;003A03	-	-	?	*Caspase-3 (Dredd), nim1?*
DK1	936	002E01-02;003C02	-	-	-	
DK1	935	002F06;003C05	-	-	-	
DK1	729	003C02-03;003E03-04	-	-	+	*N*
DK1	939	003C11-003E04	-	-	-	
DK1	940	003D06-E01;004F05	?	-	-	*rap*
DK1	944	004C15-16;005A01-02	-	-	-	*Pyp3 #2*
DK1	5705	004F05;005A13	-	-	-	
DK1	945	005A08-09;005C05-06	-	-	-	
DK1	946	005C02;005D05-06	-	-	-	*rux*
DK1	5281	005C03-10;006C03-12	-	?	?	*dx, rux*
DK1	3196	006E02;007A06	-	-	-	
DK1	948	007A02-03;007C01	-	?	-	
DK1	3221	007B02-04;007C03-04	-	-	-	
DK1	949	007D01;007D05-06	-	-	?	
DK1	950	007D10;008A04-05	-	-	-	
DK1	951	007F01-02;008C06	-	-	-	
DK1	3651	008B05-06;008D08-09 or 008D01-02;008E01-02	?	-	-	
DK1	952	008E;009C-D	-	-	?	
DK1	954	009B01-02;010A01-02	-	-	?	
DK1	3560	009F;010C03-05	-	?	-	
DK1	957	010A09;010F06-07	-	-	-	
DK1	959	010C01-02;011A01-02	-	-	-	*Pyp3 #3*
DK1	962	010F07;011D01	-	-	-	
DK1	964	011A01;011D-E	-	-	?	
DK1	967	011D-E;012A01-02	-	-	-	
DK1	966	011D01-02;011F01-02	-	-	-	*sno*
DK1	727	012A;012E or 011F10;012F01	-	-	-	
DK1	998	012D02-E01;013A02-05	-	-	-	
DK1	1039	012F05-06;013A09-B01	-	-	-	
DK1	3347	013F01;014B01	?	-	-	*CycD, sd*
DK1	125	014B08;014C01	-	-	?	
DK1	3217	014B13;015A09;035D-E	-	-	-	*ATR/mei-41*
DK1	993	014C02-04;015B02-C01	-	-	?	*ATR/mei-41*
DK1	4741	015D03;016A04-06	-	-	?	

DK1	4953	016A02;16C07-10, 015C01-D06;016F	-	-	-	
DK1	970	017A01;018A02	-	-	-	
DK1	971	018A05;018D	-	-	-	*Grip84? (18D3-6)*
DK1	972	018E01-02;020	?	-	?	
DK1	977	019F01-02;020E-F	-	-	-	*S6KII – Rps6K2*
DK1	3714	020A;020F	-	-	-	
DK2	3638	021A01;021B07-08	+	-	+	*Wos2/SBA1/HSP90, spen*
DK2	6283	021B07-C01;021C02-03	-	-	-	
DK2	3548	021B08-C01;021C08-D01	-	-	-	*Met30 (ebi)*
DK2	3084	021D01-02;022B02-03	n/a	n/a	n/a	*nim1 (CG4629)*
DK2	3133	022A02-03;022D05-E01	-	-	-	
DK2	90	023A01-02;023C03-05	-	-	-	*gammaTub23c*
DK2	97	023C03-05;023D01-02	-	-	-	
DK2	3573	23C;23E3-6 023C;023E03-06	?	-	-	
DK2	4954	023D02;023E3	-	-	-	
DK2	693	024C02-08;025C08-09	-	-	-	
DK2	3813	025A05;025E05	-	-	-	
DK2	781	025D02-04;026B02-05	-	-	-	*Mos #2*
DK2	490	025F03-026A01;026D03-11	-	-	-	
DK2	6299	026B01-02;026D01-02	-	-	-	
DK2	6338	026D03-E01;026F04-07	-	-	-	
DK2	6374	026D10-E01;027C01	?	?	?	
DK2	3571	027A;028A	-	-	-	*Rca1*
DK2	1357	027C02-09;028B03-04	-	-	-	
DK2	3077	027D-E;028C	-	-	-	
DK2	4955	028B02;028D03	-	-	-	
DK2	140	028DE (within)	-	-	-	
DK2	179	028E04-07;029B02-C01	-	-	-	
DK2	2892	029C01-02;030C08-09	-	-	-	*Suc1/Cks1 #1*
DK2	556	030C01-02;030F	-	-	-	
DK2	1045	030D-30F;031F	+	+	+	*da*, ***cdc2***
DK2	1469	031C-D;032D-E	-	?	+	*dal?*,***cdc2***
DK2	5869	032D01;032F01-03	-	-	-	*dal?, cmet*
DK2	3079	032F01-03;033F01-02	-	-	-	
DK2	3344	033B02-03;034A01-02	-	-	-	
DK2	3138	034B12-C01;035B10-C01	-	-	-	*kuz?*
DK2	3588	035B04-06;035F01-07	-	-	-	*Su(H)*
DK2	1491	035D01;036A06-07	+	-	+	*cdc25twe, grp, esg, fzy, CycE*
DK2	2583	035F-036A;036D	-	-	-	*Chk1/grp*
DK2	3180	036A08-09;036E01-02	-	-	-	
DK2	420	036C02-04;037B09-C01	-	-	-	
DK2	3189	036E04-F01;038A06-07	-	-	-	*Pyp3 #6, gammaTub37C*
DK2	167	038A06-B01;040A04-B01	-	-	?	*Chk2/Cds1/loki, neb (KLP38B)*
DK2	4959	040h35;040h38L	-	-	-	
DK2	749	041A-B;042A02-03	?	-	-	*nim1 (CG17528)*
DK2	739	041A	-	-	-	
DK2	1007	042A01-02;042E06-F01	-	-	-	
DK2	1888	042B03-05;043E15-18	-	-	-	
DK2	3368	042E;044C	-	-	?	*septin3*

DK2	198	043F;044D03-08	-	-	-	*septin1*
DK2	201	044D01-04;044F12	-	-	-	
DK2	3591	044F10;045D09-E01	-	-	-	
DK2	4966	045A06-07;045E02-03	-	-	-	
DK2	1743	046A;046C	-	-	-	*dap?*
DK2	1702	046C;047A01	-	-	-	
DK2	447	046D07-09;047F15-16	-	-	-	*14-3-3 zeta*
DK2	190	047D03;048B02	-	-	-	
DK2	1145	048A03-04;048C06-08	-	-	-	
DK2	4960	048E;049A	-	-	-	
DK2	5879	048E12-F04;049A11-B06	?	-	?	
DK2	754	049A04-13;049E07-F01	?	-	+	*sca, vg, Mos #1*
DK2	442	049C01-04;050C23-D02	-	-	-	*mam?, cnn, cables*
DK2	1896	051A01-02;051B06	-	-	-	
DK2	5422	051A05;051C01	-	-	-	
DK2	1150	051B05-11;051D07-E02	-	-	-	
DK2	3518	051D03-08;052F05-09	-	-	-	*Pyp3 #5*
DK2	3520	052F05-09;052F10-53A01	-	-	-	*Pyp3 #5*
DK2	5680	054B17-C04;054C01-04	-	-	-	
DK2	5574	054C01-04;054C01-04 (?)	-	-	-	
DK2	3064	054E08-F01;055B09-C01	-	-	-	
DK2	1547	055A;055F	-	-	-	
DK2	757	055E02-04;056C01-11	-	-	-	
DK2	543	056F05;056F015	-	-	-	
DK2	3467	056F09-17;057D11-12	?	-	-	
DK2	5246	057D02-08;058D01	-	-	-	
DK2	282	058D01;059A	-	-	-	
DK2	3909	059A01-03;059D01-04	-	-	-	*CycB?*
DK2	1682	059D05-10;060B03-08	-	-	-	
DK2	2604	060C05-06;060D09-10	-	-	-	
DK2	2471	060E02-03;060E11-12	-	-	-	
DK2	3157	060E06-08;060F01-02	-	-	-	
DK2	4961	060F01;060F05	-	-	-	
DK3	2577	061A;061D03	-	-	-	
DK3	439	061C05-08;062A08	-	-	-	*Rac1, KLP61F*
DK3	5411	062B01;062E03	-	-	-	
DK3	2400	062B08-09;062F02-05	-	-	?	
DK3	3650	062F;063D	-	-	-	*Hsp83/90*
DK3	3649	063C02;063F07	-	-	?	
DK3	3686	063F04-07;064C13-15	-	-	-	
DK3	3096	064C;065C	-	-	-	*Dhc64C*
DK3	4393	065A02;065E01	-	-	-	
DK3	1420	065F03;066B10	+	-	-	*Rac2, pbl*
DK3	1541	066B08-09;066C09-10	-	-	-	
DK3	3024	066D10-11;066E01-02	-	-	-	*dally?*
DK3	4500	066E01-06;066F01-06	-	-	-	*dally?*
DK3	1688	066F05;066F05	-	-	-	
DK3	2479	066F05;067B01	-	-	-	
DK3	997	067A02;067D07-13 or 067A05;067D09-13	-	-	alphaTub67C	
DK3	89	067E01-02;068C01-02	-	-	-	
DK3	2611	068A02-03;069A01-03	-	-	-	*CycA*

DK3	2612	068C08-11;069B04-05	-	-	-	*CycA*
DK3	4507	069B01-05;069D01-06	-	-	-	
DK3	5915	069D02;069E03-05	-	-	-	*Pyp3 #4*
DK3	4366	069F03-04;070C03-04	-	-	-	*Ly*
DK3	3124	070C01-02;070D04-05	-	-	-	
DK3	3126	070D02-03;071E04-05	-	-	-	
DK3	2992	071C;071F	-	-	-	*CDC34*
DK3	3640	071F01-04;072D01-10	-	-	-	*Chk3*
DK3	2993	072C01-D01;073A03-04	-	-	-	
DK3	2998	073A03;074F	-	-	?	
DK3	2608	075A06-07;075C01-02	-	-	-	
DK3	2990	075B08;075F01	-	-	-	*Rad9*
DK3	3000	076A03;076B02	-	-	-	
DK3	3617	076B01-02;076D05	-	-	?	
DK3	5126	076B04;077B	-	-	-	
DK3	2052	077A01;077D01	-	-	?	*Psn, trbl, polo*
DK3	3127	077B-C;077F-78A	-	-	-	
DK3	4429	077F03;078C08-09	-	-	-	*fng*
DK3	3627	078A;078E, 078D;079B	-	-	-	
DK3	4430	078C05-06;078E03-079A01	-	-	-	
DK3	4506	079C01-03;079E03-08	-	-	-	
DK3	5951	079D03-E01;079F03-06	-	-	-	
DK3	4370	079F;080A	-	-	-	
DK3	1518	081F03-06;082F05-07	-	-	-	*Hus1/Cdr2*
DK3	4787	082F03-04;082F10-11	-	-	-	
DK3	5694	082F08-10;083A01-03	-	-	-	
DK3	1990	083C01-02;084B01-02	-	-	-	
DK3	1884	084A01-02;084B01-02	-	-	-	
DK3	1842	084B01-02;084D11-12 or A06,D14	-	-	-	*alphaTub84B*
DK3	1968	084D04-06;085B06	-	-	-	*ato, Cla4/PAK, Suc1/Cks1#2*
DK3	1962	085A02;085C01-02	-	-	-	*Suc1/Cks1 #2*
DK3	1931	085D08-12;085E07-F01	-	-	-	
DK3	1893	085D11-14;085F06	-	-	-	
DK3	3128	086C01;087B01-05	-	-	-	*nim1 (Kp789)*
DK3	3003	086E02-04;087C06-07	-	-	-	
DK3	3007	087B11-13;087E08-11	-	-	-	
DK3	1534	087D01-02;088E05-06	-	-	?	*Rad17*
DK3	383	088E07-13;089A01	-	-	-	*ATM*
DK3	1467	089B07-08;089E07-08;020	-	-	-	
DK3	4431	089E01-F04;091B01-B02	-	-	?	
DK3	3071	089E03-04;090A01-07	-	-	?	
DK3	3011	090F01-F04;091F05	-	-	-	*14-3-3 epsilon*
DK3	3012	091F01-02;092D03-06	+	-	+	***Delta***
DK3	4962	092B03;092F13	-	-	-	*septin2, H, Cdk2, PP2A*
DK3	3340	093B06-07;093D02	-	+	-	*Met30 (slmb)*
DK3	2425	093B;094	-	-	-	*CAK*
DK3	4940	095A05-07;095C10-11	-	-	-	
DK3	2585	095A05-07;095D06-11	-	-	-	
DK3	4432	095D07-D11;095F15	-	-	-	
DK3	2363	095F07;096A17-18	-	-	-	

DK3	3468	096A02-07;096D02-04	-	-	-	*CycB3*
DK3	5601	096F01;097B01	-	-	+	***E(spl)***
DK3	1910	097A;098A01-02	-	-	-	
DK3	823	097E03;098A05	-	-	-	*Ser*
DK3	430	098E03;099A06-08	+	-	+	***cdc25stg***, *Pkc1/Mpk1*
DK3	669	099A01-02;099B06-11	+	-	+	***cdc25stg***, *Pyp3#1*
DK3	3547	099B05-06;099E04-F01	-	-	-	*BUB3*
DK3	3546	099C08;100F05	-	-	-	
DK4	1785	101F01;102F08	-	-	-	*Crk adaptor/p38*
DK4	759	102E02;102E10	-	-	-	

Legend:

A minus (-) sign indicates that no interaction was observed.

A plus (+) sign indicates that an interaction was observed.

A question mark (?) indicates an indeterminate result, most often due to an interaction with a balancer chromosome or a balancer chromosome with a strong eye phenotype which rendered comparison impossible.

Crosses marked n/a are not scoreable because of a strong eye phenotype produced by the deficiency.

Potential interactors are marked with a question mark (?) if their assignment to a given deficiency is ambiguous.

Potential interactors shown in bold text have been confirmed as legitimate.

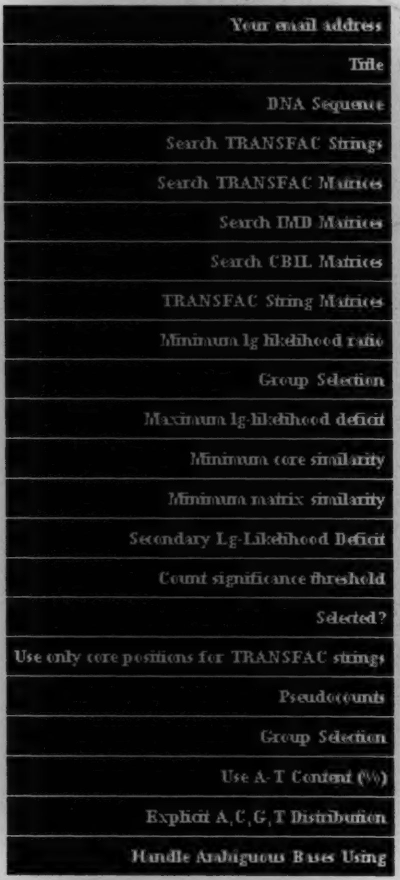
1.6 Searching for transcription factor binding sites using TESS

Obtaining DNA sequence:
Go to FlyBase on the World Wide Web (http://flybase.bio.indiana.edu/). Search terms to enable linking directly to the relevant gene are "Dwee1", "Dmyt1" and "Stg". Follow the link titled "GadFly" to reach the Berkeley *Drosophila* Genome Project (BDGP) genome annotation database profile for the gene. From the GadFly profile, open the "View on sequence (interactive gif)" link (which opens a new window) and use the zoom tool to center the gene on the sequence in a region of about 20 kb. Then select "Dump view as: FASTA" to download the surrounding genomic sequence. In the original GadFly profile window, select "Display—gene region fasta" to view the sequence of the gene and a short upstream region. Select a small segment of DNA sequence leading up to the first base of the transcript. Copy and paste this short sequence into a DNA analysis utility to generate the reverse complement (I used http://molbiol.virtualave.net/revcomp.html). The reverse complement is used so that the sequence proceeds from the first base of upstream sequence rather than the last. Use this reverse complement segment to search the genomic DNA sequence (obtained via "Dump view as: FASTA" above) to locate the transcription start site. Paste the upstream sequence into a DNA utility program (I used *BioEdit*: http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and select the desired sequence interval (in this case it was 2000 or 1000 base pairs).

Searching for transcription factor binding sites:
Paste the selected upstream sequence interval into the 'TESS: Transcription Element Search Software on the WWW' service from the University of Pennsylvania (http://www.cbil.upenn.edu/tess) using the combined search page (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=SEA-FR-Query). Statistical parameters can be modified to varying degrees of stringency (i.e. greater or less tolerance of sequence divergence from a given consensus) depending on the size of interval you want to search (note that the maximum contiguous sequence length that TESS will accept is 2000 base pairs), and the density of transcription factor binding sites within that sequence. It should also be noted that the sequence matches TESS detects are taken from the literature, and are often not true consensus sequences in the sense that they are compared to specific transcription factor binding sequences, not to a statistically rendered consensus. Search parameters were identical within each set for each gene being analyzed (e.g., Set 1: 2000 base pairs upstream of *Dwee1*, *Dmyt1* and *cdc25*^{string}. Set 2: 1000 base pairs upstream of *Dwee1*, *Dmyt1* and *cdc25*^{string}). For detailed technical specifications on the TESS search engine, altering its parameters, and the databases it queries, go to:

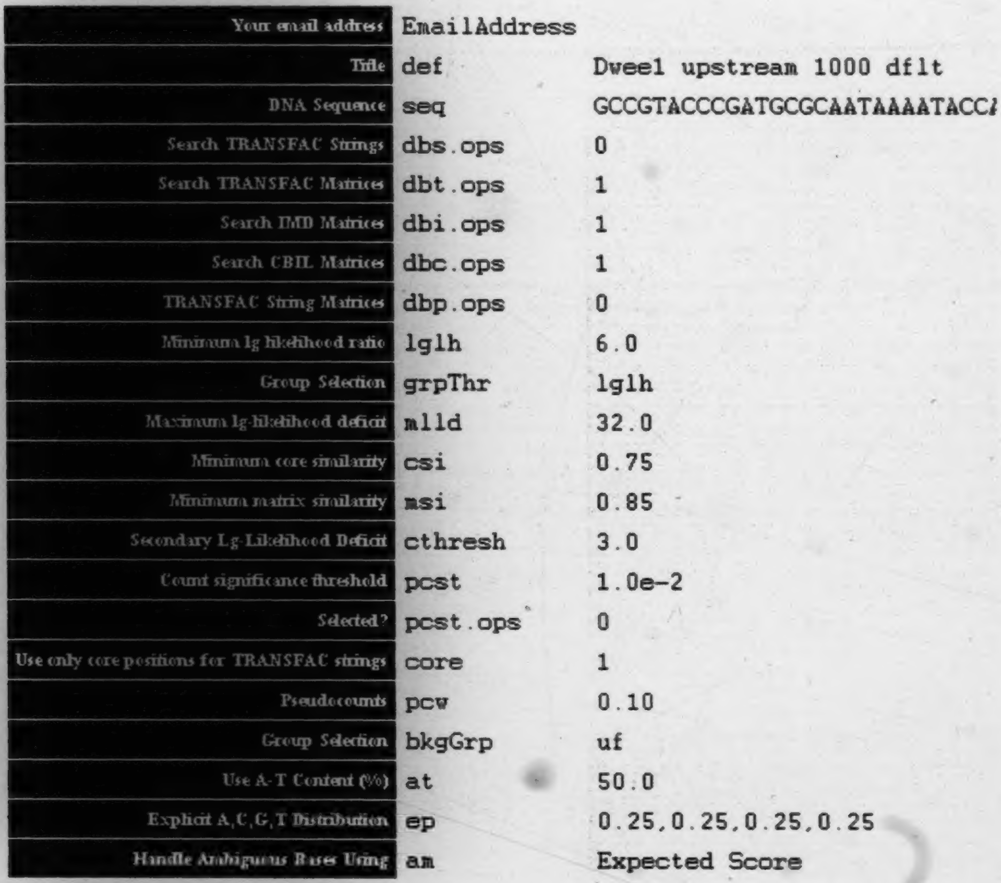
http://www.cbil.upenn.edu/tess/techreports/1997/CBIL-TR-1997-1001-v0.0.pdf

The following is a screen shot summarizing the search parameters used for the 2000 base pair upstream search:



Your email address	EmailAddress	
Title	def	Dweel 2000 upstream string
DNA Sequence	seq	GCCGTACCCGATGCGCAATAAAATACC
Search TRANSFAC Strings	dbs.ops	1
Search TRANSFAC Matrices	dbt.ops	1
Search IMD Matrices	dbi.ops	1
Search CBIL Matrices	dbc.ops	1
TRANSFAC String Matrices	dbp.ops	1
Minimum lg likelihood ratio	lglh	16
Group Selection	grpThr	lglh
Maximum lg-likelihood deficit	mlld	6
Minimum core similarity	csi	0.75
Minimum matrix similarity	msi	0.85
Secondary Lg-Likelihood Deficit	cthresh	3.0
Count significance threshold	pcst	1.0e-2
Selected?	pcst.ops	0
Use only core positions for TRANSFAC strings	core	1
Pseudocounts	pcw	0.10
Group Selection	bkgGrp	uf
Use A-T Content (%)	at	50.0
Explicit A,C,G,T Distribution	ep	0.25,0.25,0.25,0.25
Handle Ambiguous Bases Using	am	Expected Score

Search parameters used for the 1000 base pair upstream search:



Your email address	EmailAddress	
Title	def	Dweel upstream 1000 dflt
DNA Sequence	seq	GCCGTACCCGATGCGCAATAAAATACC
Search TRANSFAC Strings	dbs.ops	0
Search TRANSFAC Matrices	dbt.ops	1
Search IMD Matrices	dbi.ops	1
Search CBIL Matrices	dbc.ops	1
TRANSFAC String Matrices	dbp.ops	0
Minimum lg likelihood ratio	lglh	6.0
Group Selection	grpThr	lglh
Maximum lg-likelihood deficit	mlld	32.0
Minimum core similarity	csi	0.75
Minimum matrix similarity	msi	0.85
Secondary Lg-Likelihood Deficit	cthresh	3.0
Count significance threshold	pcst	1.0e-2
Selected?	pcst.ops	0
Use only core positions for TRANSFAC strings	core	1
Pseudocounts	pcw	0.10
Group Selection	bkgGrp	uf
Use A-T Content (%)	at	50.0
Explicit A,C,G,T Distribution	ep	0.25,0.25,0.25,0.25
Handle Ambiguous Bases Using	am	Expected Score